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11441

**Isolation of an Unpigmented Skin Reactive Constituent from
Extracts of Ragweed Pollen by Electrophoresis.***

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In 1938 experiments were undertaken, using a conventional moving boundary technique, to separate the fractions and to determine the electrical charge of the constituents of giant ragweed pollen extracts. At that time it was observed that the pigments in dialyzed ragweed extracts at pH 7.0 were negatively charged. The results during that summer and the succeeding winter, however, were contradictory, because the relation of the pigments to the biologically active constituents was uncertain.

* This investigation has been aided by a grant from the Josiah Macy, Jr., Foundation.

Using the Tiselius¹ cell for the study of the moving boundaries of protein solutions and the Philpot-Svensson² technic to analyze the quantity and nature of the constituents, further progress may now be reported. Figures 1a and 1b are illustrations of the type of curves given by the major constituent. This major constituent is negatively charged, slow moving, unpigmented, and highly skin reactive in persons sensitive to ragweed. A minor constituent, about 1/5 that of the unpigmented major constituent, appearing in the pigment moves approximately 10 times as fast as the unpigmented major one in Fig. 1a. The pigments apparently did not all migrate with the minor boundary but also moved towards the positive pole. Fig. 1b, which also illustrates as a major constituent an unpigmented component has, in addition, several minor constituents migrating towards the positive pole. The electric mobility of the unpigmented skin reactive constituent is $0.05 \mu/\text{sec}$ at 1.5°C . It is of interest to note that the electrical mobility of quartz particles in similar ragweed solutions studied by Abramson, Sookne and Moyer³ approximately agrees with this value when temperature corrections for viscosity are made.

In Fig. 2, the section of the electrophoresis cell labelled (a) shows a Schlierung pattern of the boundary between the unpigmented slow moving active constituent and the buffer; section (b) shows a lightly pigmented section; section (c) was so highly pigmented that practically no light came through; and section (d) contained the ad-



FIG. 1a.

The white area labelled US is the Philpot-Svensson curve of the unpigmented, skin reactive, slow moving constituent. The minor peak is a faster moving constituent in the pigment.



FIG. 1b.

This curve was obtained from another sample of ragweed and illustrates again the US fraction as well as four minor constituents migrating in or with the pigment.

¹ Tiselius, A., *Trans. Farad. Soc.*, 1937, **33**, 524.

² Svensson, H., *Koll. Z.*, 1939, **87**, 190.

³ Abramson, H. A., Sookne, A., and Moyer, L. S., *J. Allergy*, 1939, **10**, 317.

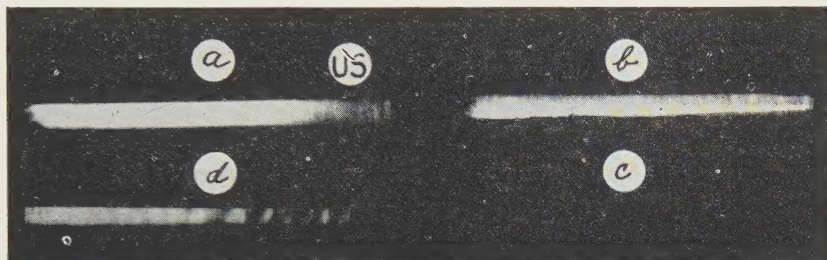


FIG. 2.

To conserve space, these Schlieren or band patterns of the constituents of extracts of ragweed pollen have been rotated 90° with the top of the electrophoresis cell on the left hand side. The US fraction is indicated by the broad band in Fig. 2a. For description of sections b, c, d, see text.

vancing column of pigmented constituents and shows many bands with electrical mobilities closely related to one another but with very low concentrations of each. These bands are essentially similar to those illustrated in Fig. 1b.

It has been previously observed by one of us that the skin reactive constituents of giant ragweed extract may be introduced by an electrical field into the skin by either the negative or the positive pole. Indeed, it was surprising to observe that the positive pole was more efficient than the negative pole with unfractionated dialyzed solutions at pH 7.0. The present studies show that there was no positively charged component observable by the method in the six dialyzed extracts thus far studied. The absence of a positively charged skin reactive constituent is evidence in favor of the point of view of Abramson and Gorin⁴ that diffusion forces primarily account for the movement of the skin reactive constituent into the skin during the passage of the current.

Further experimental evidence that the positive pole may transport a negatively charged skin reactive constituent was obtained in the following way. A sample of the unpigmented active fraction was dialyzed for one hour against distilled water to remove phosphate buffer. It was then brought by addition of sodium hydroxide to pH 7.0. The nitrogen content of this solution was 0.25 mg/cm³. Using this dialyzed negatively charged constituent sufficient ragweed was introduced by electrophoresis from the positive pole for 3 minutes to produce a severe skin reaction in an individual markedly skin-sensitive to ragweed pollen.

Some idea of the skin reactivity of the unpigmented fraction in

⁴ Abramson, H. A., and Gorin, M. H., *J. Physical Chem.*, 1939, **43**, 3; Abramson, H. A., and Gorin, M. H., *Chem. Prod.*, 1940, **3**, 37.

relation to its nitrogen content is given by the fact that a 1:1000 dilution of a solution containing 0.3 mg of nitrogen/cm³ still retained skin reactivity in a ragweed-sensitive case. That is, a solution containing 0.0003 mg of nitrogen/cm³ scratched into the skin by the usual technic, gave a positive test.

The skin reactivity of the pigmented fractions has not as yet been investigated nor have undialyzed solutions been examined electrophoretically.

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Absorption Rates and Biologic Effects of Pellets of α -Estradiol and α -Estradiol Benzoate in Women.

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In a preliminary communication, we have reported the subcutaneous implantation of crystals of α -estradiol benzoate in a group of 10 menopausal patients who had well-defined morphologic signs and symptoms of estrogen deficiency.¹ It was shown that, by this method of administering estrogens, it was possible to achieve a strikingly more prolonged effect than is obtained with comparable amounts of the hormone, administered parenterally, in solution in oil. It was subsequently demonstrated that a correspondingly prolonged inhibition of the hyperactive hypophysis occurred following the estrogen implantation.²

Since Deanesley and Parkes^{3, 4} have shown that prolonged estrogenic effects resulted from the subcutaneous implantation of pellets of estrogens in rats, we thought it desirable to study in women the duration of the physiologic and therapeutic effects of pellets as compared with crystals of the same estrogenic substance. Accordingly, 46 patients were implanted with pellets and 55 with crystals of either α -estradiol or α -estradiol benzoate. During a period of observation of approximately one year, it was noted that more prolonged physiologic and therapeutic effects re-

¹ Salmon, U. J., Walter, R. I., and Geist, S. H., *Science*, 1939, **90**, 162.

² Salmon, U. J., Geist, S. H., and Walter, R. I., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 424.

³ Deanesley, R., and Parkes, A. S., *Proc. Roy. Soc. B.*, 1937, **124**, 279.

⁴ Deanesley, R., and Parkes, A. S., *Lancet*, 1938, **2**, 606.

sulted from the implanted crystals than from pellets of similar weight and chemical constitution. We, therefore, felt it important to determine the rate of absorption of the hormone by removing and reweighing the implanted pellets at varying intervals after the implantation. At the same time, the duration of biologic effects of the implanted hormone, as manifested by morphologic changes in the endometrium and vaginal mucosa, were studied by means of repeated vaginal smears and vaginal and endometrial biopsies. Here we wish to report the results of our studies on the absorption rate and duration of biologic effects of the implanted estrogen pellets.

Material and Procedure. From the 46 cases implanted with pellets, 14 patients (9 natural menopause, 4 surgical castrates and 1 X-ray castrate) were selected for excision. The duration of the menopause, in this group, varied from 2 months to 7 years. All of the patients had either clinical or morphologic evidence of estrogen deficiency, or both, prior to the implantation.

Round, flat pellets of α -estradiol and α -estradiol benzoate,* sterilized by autoclaving (265°F, at 15 lb pressure, for 30 minutes), varying in weight from 15 to 25 mg each, were implanted, subcutaneously, in the outer aspect of the thigh. Nine patients were implanted with a single pellet; 4 with 2 pellets; and one with 3 pellets. In 8 cases, the pellets were of α -estradiol and in 6, α -estradiol benzoate. The skin was prepared with alcohol and iodine and anesthetized with 1% novocaine. The pellets were implanted, approximately $\frac{3}{4}$ of an inch below the surface into the subcutaneous fat, through a skin incision approximately $\frac{1}{2}$ inch in length. The implantation sites were excised at varying intervals after the implantation and the pellets were weighed after drying in a desiccator.

Results. Absorption Rates of α -Estradiol Pellets. The α -estradiol pellets were excised at periods of time varying from 130 to 245 days following the implantation. Each pellet was found to be closely enveloped by a fibrous capsule. Microscopic study of the surrounding tissues revealed a typical non-specific foreign body reaction. (The histologic details of the tissue reaction to the pellets are described elsewhere.⁵) The results of this study are presented in Table I.

The rate of absorption, expressed in terms of average percent

* For the α -estradiol and α -estradiol benzoate pellets used in this investigation, we are indebted to Dr. Erwin Schwenk, Schering Corporation, Bloomfield, N. J., and to Mr. Robert C. Mautner, Ciba Pharmaceutical Products, Summit, N. J.

⁵ Geist, S. H., Walter, R. I., and Salmon, U. J., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 712.

TABLE I.

Case	Age	Menopause	Post-menopausal, mo	Estrogen	Initial wt, mg	Wt on removal, mg	Duration of implantation, days	Amount absorbed, mg	Absorption per 30 days, %	Avg daily No. of rat units absorbed, R.U.	Maximum duration of therapeutic effect	Clinical status at excision
1	45	n	7	α -est	25	18.6	193	6.4	3.9	398	138 days	75% recurrence
2	50	n	36	"	15	11.0	160	4.0	5.0	300	160 plus	"
3	47	n	24	"	45*	42.2	23	2.8	8.0	1461	0	No improvement
4	42	s	6	"	15	12.0	130	3.0	4.6	277	130 plus	No recurrence
5	45	n	36	"	25	16.4	180	8.6	5.7	573	134 days	"
6	46	n	13	"	15	9.6	213	5.4	5.1	304	0	No improvement
7	49	n	60	"	25	21.1	245	3.9	1.9	191	101	100% recurrence
8	48	n	3	"	15	9.8	225	5.2	4.6 δ	277 \dagger	190	75% "
9	51	s	84	α -est-b	50 \dagger	49.4	87	0.6	0.41	41	0	No improvement
10 \dagger	27	s	4	"	25	20.7	207	4.3	2.5	125	75	100% recurrence
11	51	n	60	"	50 \dagger	48.0	101	2.0	1.2	119	60	100% "
12	50	n	24	"	25	20.4	116	4.6	4.7	238	62	100% "
13	48	x	24	"	50 \dagger	48.8	88	1.2	0.81	82	88 plus	No "
14	38	s	2	"	25	24.0	116	1.0	1.0	52	0	No improvement
					50 \dagger	46.4	156	3.6	1.4**	138 $\dagger\dagger$	45 days	100% recurrence

* 3 pellets.

† 2 "

†† This patient was implanted at 2 different times.

 δ Average of α -estradiol series = 4.85% per 30 days.** Average of α -estradiol benzoate series = 1.72% per 30 days.†† Average of α -estradiol series = 473 R.U. per day.†† Average of α -estradiol benzoate series = 113 R.U. per day.

n = natural menopause.

s = surgical menopause.

x = x-ray menopause.

 α -est = α -estradiol. α -est-b = α -estradiol benzoate.

weight loss per 30 days, varied from 1.9 to 8, with an average for the series of 4.85%. This represents, in terms of rat units, average daily absorption rates varying from 191 to 1460 R.U., with an average for the series of 473 R.U. per day. Deanesley and Parkes, in their study of α -estradiol pellets in rats, reported 6% to 9% average absorption per month.

Absorption Rates of α -Estradiol Benzoate Pellets. The α -estradiol benzoate pellets were excised at intervals varying from 87 to 207 days after the implantation. In this series, also, a fibrous capsule was found surrounding each pellet. The rate of absorption in terms of average percent weight loss per 30 days, varied from 0.41 to 4.7. The average rate of absorption (per 30 days), of this series, was 1.72%. This represents, in terms of rat units, variations from 41 to 238 R.U. per day, with an average for the series of 113 R.U. per day.

Biologic Effects of α -Estradiol Pellets. All cases showed characteristic morphologic evidence of estrogenic effect, as indicated by proliferative response in the vaginal mucous membrane (smears and biopsies) and/or the endometrium, within 2 weeks after the implantation. Details of the effect of implanted estrogens upon the mucous membranes of the genital tract are being reported elsewhere.

At the time of removal of the implanted α -estradiol pellets, there was evidence (in all but 3 cases) of morphologic regression to the pre-implantation status, indicating varying degrees of estrogen deficiency. Apparently, at the time of excision, so little of the hormone was being absorbed that no estrogenic effect was demonstrable in the uterine or vaginal mucosa. In all of these cases the pellets had been retained for 180 days or more.

The 3 cases in this series that revealed morphologic evidence of continued estrogen activity, at the time of excision, had all been implanted for shorter periods of time (23, 130 and 160 days).

Biologic Effects of α -Estradiol Benzoate Pellets. In the α -estradiol benzoate series, morphologic studies, at the time of excision (87 to 207 days post-implantation), revealed, in all cases, regression to the pre-implantation status, indicating the cessation of estrogen activity.

Clinical Effects of α -estradiol Pellets. One patient (Case No. 6) experienced no relief of symptoms; 4 were relieved for periods varying from 101 to 190 days; 2 (Case No. 2 and Case No. 4) were still symptom-free at the time of excision (160 and 130 days post-implantation).

Clinical Effects of α -Estradiol Benzoate Pellets. Two patients (Case No. 9 and Case No. 13) experienced no relief of symptoms following the implantation; 5 were relieved for periods varying

from 45 to 75 days; one (Case No. 12) was symptom-free at the time of excision, which was 88 days after the implantation. It is apparent from this study that pellets of α -estradiol have a more prolonged therapeutic and biologic effect than pellets of α -estradiol benzoate.

It is important to note that despite the presence of sizeable pellets (weighing 11 to 46.4 mg) in the subcutaneous tissue, the majority of the patients, at the time of excision, exhibited clinical as well as morphologic evidence of estrogen deficiency. The fact has already been mentioned that the pellets were found to be completely enveloped by a tight, fibrous capsule.⁵ Apparently the capsule acts as a barrier, progressively retarding absorption of the hormone and reducing it finally to a level at which no demonstrable estrogen effect is exerted, either clinically or morphologically.

The question may be raised as to whether the hormone may not be inactivated by its prolonged contact with the subcutaneous tissues. Such a qualitative change in the pellets has been considered and apparently ruled out by demonstrating that the excised pellets, when reimplanted in rats, produced characteristic estrogen effects.

It is evident from this study that, in spite of the striking initial morphologic and therapeutic effects produced by the implanted estrogen pellets, there is a serious objection to this method of administering estrogens clinically, since the therapeutic effect is relatively short-lived and the patients, thereafter, retain sizeable pellets without deriving any therapeutic benefit from them.

Summary and Conclusions. A comparative study of the absorption rates and duration of biologic effectiveness of implanted pellets of α -estradiol and α -estradiol benzoate was made in a series of 14 cases. This study revealed that the average percent absorption rate, per 30 days, of α -estradiol pellets was 4.85% (= 473 rat units per day), as compared to 1.72% (= 113 rat units per day) for pellets of α -estradiol benzoate. The duration of biologic and therapeutic effects was definitely longer in the α -estradiol series. It is concluded, on the basis of these studies, that the fibrous capsule which forms about the pellets progressively decreases the rate of absorption of the hormone, so little being absorbed finally that no demonstrable morphologic or therapeutic effect is produced. Furthermore, because of the fact that absorption of the hormone in effective amounts ceases when only a relatively small amount of the pellet has been absorbed, it is concluded that the implantation of pellets (weighing 15 to 25 mg) of α -estradiol and α -estradiol benzoate is not a satisfactory method of administering estrogens clinically.

We wish to gratefully acknowledge the technical assistance of Mr. A. A. Salmon.

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Inhibitory Action of Testosterone Propionate on the Human Ovary.

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In previous communications,^{1, 2, 3} it has been shown that, by the administration of adequate amounts of testosterone propionate to cyclical women, menstruation can be suppressed and the secretory phase of the endometrium abolished, resulting in hypoplasia or atrophy of the endometrium. These effects of testosterone propionate were interpreted as indicating inhibition of ovulation (probably mediated through the hypophysis) with consequent suppression of estrogen and progesterone formation.

In animals, some workers have reported that synthetic androgens have a stimulating action upon the ovaries of rats⁴⁻⁸ and mice.⁹ Others have reported inhibition of ovulation in rabbits¹⁰ and monkeys¹¹ and ovarian atrophy in rats.^{12, 13}

In the study reported here, an attempt was made to determine what effect testosterone propionate has upon the ovaries of cyclical women and to correlate the ovarian response with the endometrial and vaginal changes.

Two regularly cyclical women, requiring exploratory laparotomy, were selected for this study. Endometrial biopsies were taken

¹ Gaines, J. A., Salmon, U. J., and Geist, S. H., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 779.

² Geist, S. H., Salmon, U. J., and Gaines, J. A., *Endocrinology*, 1938, **23**, 784.

³ Salmon, U. J., Geist, S. H., and Walter, R. I., *Am. J. Obs. and Gyn.*, 1939, **38**, 264.

⁴ Korenchevsky, V., Dennison, M., and Hall, K., *Biochem. J.*, 1937, **31**, 780.

⁵ Wolfe, J. M., and Hamilton, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1937, **37**, 189.

⁶ McKeown, T., and Zuckerman, S., *Proc. Roy. Soc., London, s.B.*, 1937, **124**, 362.

⁷ Salmon, U. J., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 352.

⁸ Nathanson, I. T., Franseen, C. C., and Sweeney, A. R., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1938, **39**, 384.

⁹ Starkey, W. F., and Leatham, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1938, **39**, 218.

¹⁰ Cotte, G., Martin, J. F., and Mankiewicz, E., *Gynecologie*, 1937, **36**, 561.

¹¹ Zuckerman, S., *Lancet*, 1937, **2**, 676.

¹² McEuen, C. S., Selye, H., and Collip, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1937, **36**, 390.

¹³ Mazer, M., and Mazer, C., *Endocrinology*, 1939, **24**, 175.

before and during the period of testosterone propionate administration, at intervals of 1 to 2 weeks. Vaginal smears were taken 3 times weekly. Testosterone propionate was administered for 31 and 15 days, respectively, the total amounts being 925 and 1,225 mg. The histologic findings of the ovaries were correlated with the endometrium and vaginal smears. A resumé of the protocols follows:

Case I. Age 29. Gravida 1. Para 0. Menses lasting 5 days occurred at regular intervals of 4 weeks. A preliminary endometrial biopsy, taken during the menstrual period, revealed the presence of secretory changes. It was assumed, therefore, that an ovulatory cycle had just been completed. Preliminary vaginal smears were of the normal physiologic type. Testosterone propionate* injections were started 3 days pre-menstrually and continued, at 1 to 3 day intervals, until 925 mg had been administered over a period of 31 days. Menstruation failed to take place by the 34th day of the cycle, at which time operation was performed. At this time, the endometrium was reduced to a state of hypoplasia, while the vaginal smears revealed typical estrogen deficiency characteristics. The ovaries showed no gross evidence of a mature follicle or recent corpus luteum. Two longitudinal sections were made through the entire width of each ovary down to the hilus and the central wedges removed for histologic study. Microscopic examination revealed the presence of small, collapsed or cystic corpora lutea of previous cycles, but no maturing follicles or current corpus luteum.

Case II. Age 47. Gravida 2. Para 2. Menses had occurred quite regularly at 26 to 28 day intervals, lasting for 5 to 6 days. The patient entered the hospital because of lower abdominal pain related to uterine fibromyomata. An endometrial biopsy, taken pre-menstrually, revealed a typical secretory phase. Preliminary vaginal smears revealed a normal estrogen effect. During the first 16 days of the next cycle, 1,225 mg of testosterone propionate was administered. A supravaginal hysterectomy and bilateral salpingo-oophorectomy was performed on the 17th day. The endometrium showed moderate proliferation and a complete absence of secretory phenomena. The vaginal smear, at this time showed early signs of regression. Microscopic sections of the ovaries failed to reveal any signs of a recent corpus luteum or maturing follicle.

Summary and Conclusions. Two women with regular menstrual

* For the testosterone propionate used in this investigation, we are indebted to Dr. Erwin Schwenk, Schering Corporation, Bloomfield, N. J. (Oreton), and to Mr. Robert C. Mautner, Ciba Pharmaceutical Products, Summit, N. J. (Perandren).

cycles were injected with testosterone propionate (925 and 1,225 mg), in order to determine whether ovulation could be inhibited. In one patient, the ovaries, examined on the 34th day of the cycle, showed no evidence of a recent corpus luteum or mature graafian follicle. In the second patient, examination of the ovaries, on the 17th day of the cycle, did not reveal any evidence of ovulation. In the latter case, while ovulation might have occurred after the 17th day, it was deemed unlikely in an individual with a regular 26 to 28 day cycle.

It appears from this study that testosterone propionate, if administered in adequate amounts to the cyclical human female, can inhibit full follicle maturation, ovulation and corpus luteum formation, associated with regressive changes in the endometrium and vaginal mucosa. The question arises as to whether the testosterone propionate acts directly upon the follicular apparatus or indirectly through inhibition of the gonadotropic activity of the pituitary. In view of the fact that testosterone has been shown to suppress the gonadotropic activity of the hypophysis in post-menopausal women^{14, 15} and rats,¹⁶⁻¹⁸ it is logical to conclude that the inhibitory effect of testosterone propionate upon the human ovary is mediated through the pituitary.

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Effectiveness of Sulfanilamide upon Anaerobic Hemolytic Streptococci.

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(Introduced by J. A. Kolmer.)

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Previous work in this laboratory¹ has indicated that on primary isolation a significant proportion of hemolytic streptococci are incapable of developing upon the surface of aerobic, infusion blood-

¹⁴ Salmon, U. J., *Proc. Soc. Exp. Biol. and Med.*, 1937, **37**, 488.

¹⁵ Nathanson, I. T., and Towne, L. E., *Endocrinology*, 1939, **25**, 754.

¹⁶ Nelson, W. O., and Gallagher, T. F., *Anat. Rec.*, 1935, **64**, 129.

¹⁷ Wolfe, J. M., and Hamilton, J. B., *Endocrinology*, 1937, **21**, 603.

¹⁸ Allanson, M., *Proc. Roy. Soc., London, s.B.*, 1937, **125**, 196.

¹ Spaulding, E. H., and Goode, W., *J. Lab. and Clin. Med.*, 1939, **25**, 305.

agar plates. Although most of these "anaërobic" isolations become quickly adapted to aërobic cultivation (temporarily anaërobic), a small percentage persist as obligate anaërobes. As a result of the clinical observation that several patients infected with obligately anaërobic hemolytic streptococci (Group A) responded poorly to sulfonamide-therapy, two such strains were selected for experimental study. The results are being reported because of the increasing interest in the relationship between anaërobiosis and sulfanilamide activity.²⁻⁵

Both strains reacted with Group A antiserum, and fermented trehalose but not sorbitol. Strain S, originating from a case of bronchiectasis, was unable to grow on aërobic blood-agar for 18 months. The other (1097) was isolated from a hand lesion. After 14 months it began to develop aërobically. Strain S was characterized by mucoid colonies on blood agar, whereas those of 1097 were of the smooth type. On benzidine blood agar⁷ both cultures gave rise to black colonies within two hours after removal from anaërobic jar.

Experimental infection in mice was produced with considerable difficulty since only after prolonged passage did death regularly follow the injection of several million cells. It should be noted that streptococcal strains of low virulence do not usually show marked response to sulfanilamide in mice,⁶ presumably because of the large number of organisms in the inoculum. Infection was produced intraabdominally and the drug administered subcutaneously in 10 mg doses per 25 g body weight. The results are summarized in Table I.

It will be noted that neither treatment-schedule was intensive. Nevertheless, it seems evident that infection induced by one strain (S) was definitely refractory to treatment, whereas infection with the other strain (1097) was moderately susceptible to sulfanilamide therapy. Comparative experiments concerning the effect of fresh preparation of sulfanilamide and samples of the drug oxidized by exposure to air for 30 days yielded similar results.

² Fox, C. L., German, B., and Janeway, C. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **40**, 184.

³ Warren, J., Street, J. A., and Stokinger, H. E., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **40**, 208.

⁴ Shinn, L. E., Main, E. R., and Mellon, R. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **40**, 640.

⁵ Broh-Kahn, R. H., *Science*, 1939, **90**, 543.

⁶ Marshall, E. K., Jr., *Physiol. Rev.*, 1939, **19**, 240.

⁷ MacLeod, C. M., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 215.

TABLE I.
Therapeutic Effect of Sulfanilamide in Mice upon Anaerobic Hemolytic Streptococci.

Strain	No. of organisms		Lethal doses	Drug	Death of mice in days							Survived	Autopsy cultures	
	No. of mice	injected (millions)			1	2	3	4-6	7-10	11-25	Aëro.		Anaëro.	
S	45	± 25	5-10	Treated*	34	9	2						—	+
S	20	± 25	5-10	Controls	17	3							—	+
S	18	80	10	Treated†	9	8	1						—	+
S	18	80	10	Controls	18								—	+
S	30	16	5-10	Treated*	25	3	2						—	+
S	5	16	5-10	Controls	5								—	+
1097	23	3.4	5-10	Treated*	1	2	5	3	3	1		8	—	+
1097	13	3.4	5-10	Controls	11	2							—	+
1097	17	88	5-10	Treated†	6	2	2		1			6	†† 9†	+
1097	7	88	5-10	Controls	7								††	+
1097	15	6	10	Treated*	1		3	2	3	1		5	—	+
1097	5	6	10	Controls	4	1							—	+

Treatment:

*20 mg/25 g body wt daily for 6 days; 10 mg for 5 days (10 mg doses).

†10 mg/25 g body wt daily for 12 days (10 mg doses).

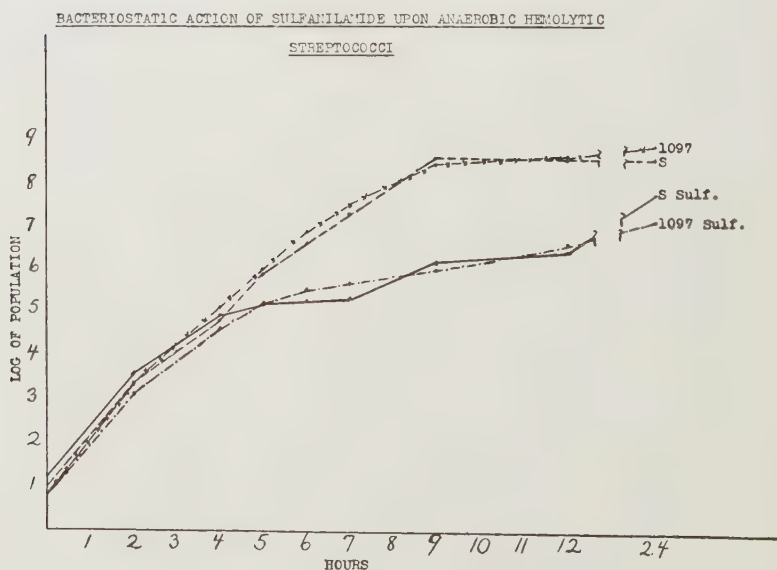
‡Occasional minute colony; aerobic subculture negative.

The *in vitro* experiments of Fox, *et al.*,² and of Warren, *et al.*,³ suggest that sulfanilamide would be ineffectual against obligately anaërobic strains. The *in vivo* results with strain 1097, however, indicate that anaërobiosis *per se* may not be an important factor in determining susceptibility of hemolytic streptococci to sulfanilamide-therapy.

In vitro bacteriostasis. Since there appeared to exist unequal responses to sulfanilamide between the 2 strains in mice, it was considered possible that *in vitro* tests might bring to light some essential difference between the two organisms. Bacteriostatic tests were performed by adding to 17 cc of peptone-free, 25% serum, infusion broth 2 cc of sulfanilamide to make a final concentration of 1:10,000. The medium was freshly prepared and incubated overnight, anaërobically. One cc of a diluted 15-hour broth culture was added with the introduction of as little oxygen as possible. Platings were made after 2, 4, 5, 6, 7, 9, 12 and 24 hours' incubation anaërobically at 37° C. At the same time the gross turbidity was estimated by barium sulfate standards and the average number of cocci per chain determined microscopically. The graphic results appear in Figure I.

Unlike the results of the mouse experiments both strains were inhibited by sulfanilamide *in vitro*. Since it is well known that in

Figure I



broth containing this drug streptococci produce long chains, the average number of cocci per chain was determined at the time each plating was made. The error due to interpreting the failure of the organisms to divide as true bacteriostasis would have been slight, however, in this instance. There were only 3 times as many elements in the drug-broth as in the control tubes, while bacteriostasis as determined by platings was in the order of a two-hundred fold difference at 9 hours.

Phagocytic Experiments. The discrepancy between the drug-resistance of strain S *in vivo* and its susceptibility *in vitro* was attributed to the enormous difference in the number of bacteria used in the two types of experiments. In mice the number was very large; in the test tube it was small. Lockwood¹⁰ has demonstrated the antibacteriostatic effect of large inocula *in vitro*. Nevertheless, it occurred to us that this strain might show marked resistance to phagocytosis in the presence of sulfanilamide. Therefore, a series of 3 *in vitro* phagocytic experiments was carried out. Using one set of cultures throughout, the test conditions were varied so as to include the use of organisms previously exposed to 1:10,000 sulfanilamide and others not subjected to the drug. The tests were incubated in the water bath at 37° C. with constant rotation. Smears were made after 30 minutes and one hour. Phagocytic activity was estimated by counting 200 leucocytes stained by Wright's method and by determining microscopically the average number of cocci per phagocyte.

A detailed description of the technique is not warranted since it was found that neither strain was markedly influenced by the presence of the drug. Strain 1097 was, perhaps, more readily phagocytized, with or without the drug, than was the S strain.

Further attempts to differentiate strains S and 1097. Because the results in mice had indicated that the strains behaved differently toward sulfanilamide, a series of fermentative tests and dehydrogenase determinations was conducted. The fermentative capacities were similar, however, and dehydrogenase studies, patterned after MacLeod⁷ were essentially negative.

Adaptation to aerobic cultivation. After 14 and 18 months respectively strains S and 1097 both developed spontaneously the ability to grow on aerobic blood agar. Since this adaptation must have been accomplished through the acquisition of new respiratory systems, it was thought desirable to determine whether a change in

¹⁰ Lockwood, J. S., *J. Immunol.*, 1938, **35**, 155.

drug susceptibility had likewise occurred. Therefore, the mouse tests were repeated using aërobic broth cultures as inoculum. Mouse-virulence was again attained with considerable difficulty and each strain tested in 30 mice receiving 20 mg of sulfanilamide per day and approximately the same number of lethal doses of culture as in Table I. No animal survived beyond the sixth day. Infection with strain S, which previously had been resistant to therapy, was now slightly less refractory. On the other hand infection with the 1097 strain, moderately susceptible when produced by the anaërobic variant, became definitely more resistant, so that the results with both strains were strikingly similar. It would appear, then, that the inability of streptococci to grow on aërobic blood agar may not necessarily be correlated with drug resistance in mice. In fact, strain 1097 was more susceptible to sulfanilamide when it was an obligate anaërobe.

Discussion. Bliss, Long and Feinstone⁸ record anaërobic non-hemolytic streptococci as refractory to sulfanilamide both *in vitro* and *in vivo*. This opinion is in agreement with the clinical experience of Colebrook and Purdie.⁹ The results with strain 1097 may be of interest, then, by suggesting the possibility that at least some strains of anaërobic hemolytic streptococci are amenable to sulfanilamide therapy.

The *in vitro* experiments of Shinn, *et al.*,⁴ with Type I pneumococcus show that the gradual reduction of the oxygen concentration to 0.04% is accompanied by a corresponding decrease in bacteriostasis by sulfanilamide. With further reduction of oxygen, however, growth inhibition reappeared. It is possible that a similar mechanism is operating in our tests with the 1097 strain. By the same token strain S may possess a different respiratory mechanism making it resistant to the drug.

Summary. 1. Two weakly virulent strains of "anaërobic," Group A, hemolytic streptococci were subjected to *in vivo* and *in vitro* tests with sulfanilamide. 2. One strain was resistant, the other moderately susceptible, to the drug in mice. 3. No essential difference between the strains could be demonstrated, however, by *in vitro* bacteriostatic, phagocytic and biochemical tests. 4. Following adaptation to aërobic incubation (14 and 18 months) both strains were refractory in mice. 5. The results indicate that anaërobiosis, *per se*, was not the fundamental factor in determining drug response of these "anaërobic" hemolytic streptococci.

⁸ Bliss, E. A., Long, P. H., and Feinstone, W. H., *So. Med. J.*, 1938, **31**, 303.

⁹ Colebrook, L., and Purdie, A. W., *Lancet*, 1937, **2**, 1237.

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Serum Phosphatase, Calcium and Phosphorus Values in Infancy.*

DONALD J. BARNES AND BERTHA MUNKS. (Introduced by Arthur H. Smith.)

From Harper Hospital, Detroit, Mich.

Investigators who have been studying diagnostic procedures and standards for judging the healing or development of early rickets in infants have commented on the difficulty of making accurate judgments when these must be based upon the physical findings, roentgenological examinations, the serum calcium and serum phosphorus determinations. We have previously cited¹ our belief that, through the determination of the serum phosphatase, we have a more accurate means of recognizing the early development of the disease. In this we simply agree with other investigators^{2, 3} and offer additional supporting evidence. However, with our interest in this abnormal state in the infant, we were struck by the lack of any considerable data defining the *normal* serum phosphatase for the infant in the first year of life. The data of Jeans and Stearns⁴ show that the plasma phosphatase, which is low at birth, rises abruptly to a maximum during the first month, maintains this peak only a short time, and then falls rather rapidly during the second or third month, gradually declining through the remainder of the year, although the phosphatase level remains higher than that found in older age groups.

Our data, based on 630 observations made upon infants during the first year of life, do not entirely coincide with those of Jeans and Stearns but we feel that they represent a good sampling of population of this age for this section of the country. Part of the patients, differentiated as "Harper Babies", upon whom 390 observations were made, were born at Harper Hospital and were followed in our outpatient clinic. They received adequate amounts of milk, vitamin D

* This study was made possible by a grant from the Upjohn Company. The assistance of the Department of Obstetrics is also acknowledged.

¹ Barnes, D. J., and Carpenter, M. D., *J. Pediatrics*, 1937, **10**, 596.

² Bodansky, A., and Jaffe, H. L., *Arch. Int. Med.*, 1934, **54**, 88; *Am. J. Dis. Child.*, 1934, **48**, 1268.

³ Morris, N., Stevenson, M. M., Peden, O. D., and Small, J., *Arch. Dis. Childhood*, 1937, **12**, 45.

⁴ Stearns, G., and Warweg, E., *J. Biol. Chem.*, 1933, **102**, 749.

as cod liver oil, and accessory foods as their ages warranted. Where there was any evidence of developing anemia, iron was added to the dietary. Any infants who showed signs or symptoms of rickets, either physical or as judged roentgenologically, were dropped from the group. The average serum phosphatase from these patients did not show the peak rise during the first month noted by Jeans and Stearns.

A second group of patients, known as "Welfare Babies", on which 240 of the observations were made, were selected from those sent to our clinic from Child Welfare stations because they were thought to be rachitic and in need of treatment. From the large number sent, those were selected who were roentgenologically negative and

TABLE I.

Age groups	"Harper babies"			"Welfare babies"*		
	No. cases	Mean, mg per 100 cc serum	Standard deviation, mg	No. cases	Mean, mg per 100 cc serum	Standard deviation, mg
(a) Statistical evaluation of calcium data.						
0 - 3 days	20	11.3	.83	—	—	—
3 - 15 "	17	10.8	1.09	—	—	—
1½ - 1½ mo	31	12.1	.70	8	11.9	.80
1½ - 2½ "	28	11.6	.71	30	11.9	.67
2½ - 3½ "	28	11.9	.62	27	11.9	.69
3½ - 4½ "	33	12.1	1.09	23	12.1	.92
4½ - 5½ "	34	11.8	.66	23	12.1	.77
5½ - 6½ "	27	11.9	.63	21	12.0	.63
6½ - 7½ "	26	11.8	.92	28	11.5	.74
7½ - 8½ "	31	11.9	.79	21	11.9	.59
8½ - 9½ "	25	11.9	.67	15	11.9	1.13
9½ - 10½ "	19	12.1	.64	18	12.0	.62
10½ - 11½ "	13	11.8	.58	12	12.1	.50
11½ - 12½ "	22	11.9	.65	10	12.3	.61
Total	354			236		
(b) Statistical evaluation of phosphorus data.						
0 - 3 days	45	6.4	.99	—	—	—
3 - 15 "	23	6.8	.75	—	—	—
1½ - 1½ mo	33	6.4	.56	8	6.6	.58
1½ - 2½ "	30	6.7	.78	30	6.3	.58
2½ - 3½ "	28	6.5	.77	27	6.3	.52
3½ - 4½ "	33	6.3	.93	23	6.1	.51
4½ - 5½ "	34	6.3	.71	23	6.4	.51
5½ - 6½ "	27	6.4	.61	22	6.2	.51
6½ - 7½ "	26	6.2	.51	28	6.3	.42
7½ - 8½ "	31	5.9	.62	21	6.1	.45
8½ - 9½ "	26	5.9	.59	15	6.2	.49
9½ - 10½ "	19	6.1	.54	21	6.1	.43
10½ - 11½ "	13	5.9	.87	12	6.4	.54
11½ - 12½ "	22	5.8	.39	10	6.4	.59
Total	390			240		

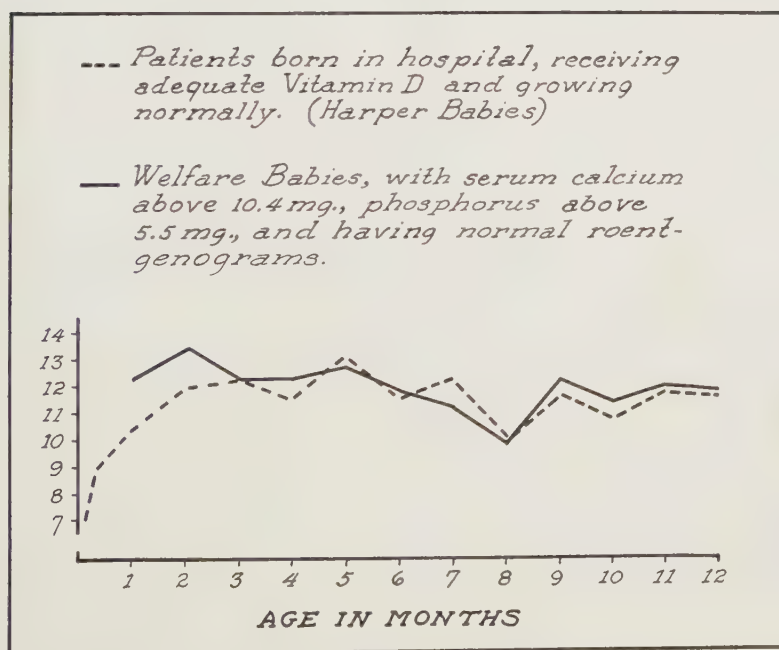
*Patients had a serum calcium of at least 10.4 mg per 100 cc, and a serum phosphorus of 5.5 mg or higher.

who were found to have a serum calcium of at least 10.4 mg per 100 cc and a serum phosphorus of 5.5 mg or above, values which are considered well above the minimal normal levels. From this group, consisting of colored and white babies, we obviously could not get data at birth and relatively few were seen during the first month. Some of them had had vitamin D, generally in small amounts.

The serum calcium, phosphorus and phosphatase were determined on blood drawn from the femoral vein. Clark and Collip's modification of the Kramer, Tisdall method⁵ was used for calcium determinations, and Bodansky's method⁶ for phosphorus and phosphatase determinations.

Table I shows that the calcium values from birth to 3 days for "Harper Babies" fell from an average of 11.3 mg per 100 cc serum to 10.8 mg during the second week, then rose to an average of 12.1 mg at the first month and were maintained rather consistently through the year. Phosphorus values averaged 6.4 mg per 100 cc serum for birth to 3 days of life and rose to an average maximum

SERUM PHOSPHATASE VALUES DURING INFANCY
(units per 100 cc.)



⁵ Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, **63**, 461.

⁶ Bodansky, A., *J. Biol. Chem.*, 1932, **99**, 197; *ibid.*, 1933, **101**, 93.

of 6.8 mg during the second week, receded slightly with the upswing of serum calcium at one month and then established a fairly constant though slightly falling value during the remainder of the year, to an average of 5.8 mg at 12 months. The "Welfare Babies" gave values which corresponded remarkably closely to those of the "Harper Babies."

The average serum phosphatase of the "Harper Babies", from birth to 3 days of age (Chart), has a value of 7.1 units per 100 cc. It shows no peak during the first month but rather a sharp rise to 11.9 units during the first 2 months, a very slow continuance of this rise through the fourth and fifth months to an average value of 13.0 units and then a gradual, slight decline through the rest of the year to a level of 11.5 units. There is a rather pronounced decline at the eighth month to a value of 9.9 units. The data, representing averages, together with standard deviations for the phosphatase values are shown in Table II.

It is noteworthy that the group labeled "Harper Babies" does not have the high average phosphatase levels at the first and second months reached by the "Welfare" patients. This probably is explained by the fact that the "Welfare Babies" were sent in as being possible cases of active rickets and their serum phosphatase values actually were elevated during the first and second months. In other words, some did have this deviation from the normal. It has been

TABLE II.
Statistical Evaluation of Phosphatase Data.

Age groups	"Harper babies"			"Welfare babies"*		
	No. cases	Mean, units per 100 cc serum	Standard deviation, units	No. cases	Mean, units per 100 cc serum	Standard deviation, units
0 - 3 days	45	7.1	2.66	—	—	—
3 - 15 "	23	8.9	2.39	—	—	—
1½ - 1½ mo	33	10.4	3.05	8	12.3	3.84
1½ - 2½ "	30	11.9	3.01	30	13.5	3.25
2½ - 3½ "	28	12.1	2.52	27	12.3	2.87
3½ - 4½ "	33	11.6	2.76	23	12.2	3.15
4½ - 5½ "	34	13.0	2.92	23	12.8	2.81
5½ - 6½ "	27	11.4	2.88	22	11.5	2.56
6½ - 7½ "	26	12.3	2.44	28	11.1	2.51
7½ - 8½ "	31	9.9	2.64	21	9.8	3.29
8½ - 9½ "	26	11.6	2.58	15	12.2	3.18
9½ - 10½ "	19	10.8	3.11	21	11.0	3.58
10½ - 11½ "	13	11.8	2.56	12	12.0	3.84
11½ - 12½ "	22	11.5	2.87	10	11.7	3.21
Total	390			240		

*Patients had a serum calcium of at least 10.4 mg per 100 cc, and a serum phosphorus of 5.5 mg or higher.

pointed out that this change in serum phosphatase is probably our earliest reliable sign of rachitic activity. Later on in the infant's life there was not the same relationship between slight physical signs and this evidence of activity. Beginning with the third month and continuing through the year, the phosphatase values for the "Welfare" group correspond fairly closely to those of the "Harper" group. We feel that the "Harper" group represents a sufficiently large, well controlled series so that we have not combined it with the "Welfare" group, rather showing each separately. As would be expected, our standard deviations are smaller in the "Harper" group than in the "Welfare" group.

The findings include both summer and winter values. Among the "Harper Babies," 170 cases tested between June 1 and November 1, constituting summer values, averaged 10.8 units for the year; while 220 cases observed from November 1 to June 1 in the winter grouping, averaged 11.0 units. During the first 6 months of life, the winter values of the "Harper Babies" averaged 1.3 units higher than the summer values, while from 6 months to one year of age, the summer values averaged 1.6 units greater than the winter values. There was not a sufficiently regular distribution of summer and winter cases in the "Welfare" group to warrant such comparisons.

These data represent, first, a series of serum phosphatase, calcium and phosphorus observations upon normal infants (Harper Babies) which should help to establish normal serum phosphatase values through the first year of life; second, a comparison with a group (Welfare Babies) which shows a slightly abnormal elevation of serum phosphatase at the first and second months but which, as judged by other serological and roentgenological standards, was normal. This supports the view that the serum phosphatase elevation is the earliest diagnostic sign of rickets, since this latter group was sent to us representing possible early cases of the disease. Furthermore, the "Welfare" group refutes, since it had such small amounts of vitamin D, the possible argument that the ingested vitamin D which was had in moderate amounts by the "Harper Babies" depressed the level of serum phosphatase below the physiological normal. In addition, further correlated data are presented concerning the serum calcium and phosphorus values during infancy.

Micromelia in Adult Fowl Caused by Manganese Deficiency During Embryonic Development.

C. D. CASKEY* AND L. C. NORRIS.

From the Department of Poultry Husbandry, Cornell University, Ithaca, N. Y.

Byerly, Titus, Ellis and Landauer¹ and Landauer² reported the occurrence of micromelia of nutritional origin in chicken embryos and in newly hatched chicks. Later Lyons and Insko³ observed micromelia in the embryos and newly hatched chicks of hens fed a diet deficient in manganese which was strikingly similar to that described by Byerly and associates and by Landauer. Lyons and Insko prevented the development of the abnormally shortened leg and wing bones by injecting manganese sulfate into the eggs just before placing them in the incubator.

Caskey, Gallup and Norris⁴ prevented the development of the embryonic reduction in bone length due to manganese deficiency by feeding the hens manganous carbonate. They also showed that one of the chief symptoms of perosis, which develops in normal chicks fed a manganese-deficient diet, is a reduction in the length of the leg and wing bones. Caskey and Norris⁵ found that perosis fails to develop when normal chicks fed a manganese-deficient diet are injected intraperitoneally with manganous chloride. Lyons and Insko³ and Gallup and Norris⁶ reported that the eggs of hens fed a manganese-deficient diet contain markedly less manganese than those of hens fed an adequate diet.

In further studies conducted at this laboratory it was found that frequently the chicks which hatched from the eggs of hens fed a low-manganese diet (0.00063% manganese) were ataxic. A group of 15 of these chicks, most of which were also micromelic, was placed in appropriate quarters for observation. During the first 8 weeks they were fed an adequate diet composed of 44.73% ground yellow corn, 20% degerminated yellow corn meal, 15% dried skim

* Cooperative G.L.F. Exchange Fellow.

¹ Byerly, T. C., Titus, H. W., Ellis, N. R., and Landauer, W., *PROC. SOC. EXP. BIOL. AND MED.*, 1935, **32**, 1542.

² Landauer, W., *Anat. Rec.*, 1936, **64**, 267.

³ Lyons, Malcolm, and Insko, W. M., Jr., *Ky. Agr. Exp. Sta. Bul.* 371, 1937, 61.

⁴ Caskey, C. D., Gallup, W. D., and Norris, L. C., *J. Nutr.*, 1939, **17**, 407.

⁵ Caskey, C. D., and Norris, L. C., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **40**, 590.

⁶ Gallup, W. D., and Norris, L. C., *Poul. Sci.*, 1939, **18**, 83.

milk, 10% meat scrap, 5% dehydrated alfalfa meal, 2.5% casein, 1.5% steamed bone meal, 0.5% calcium carbonate, 0.5% iodized salt, 0.25% cod liver oil (400 D per g) and 0.02% manganous carbonate. This diet contained 1.8% calcium, 0.95% phosphorus and 0.01% manganese. After 8 weeks the chicks were fed a somewhat similar diet containing 0.005% manganese.

Eleven of these chicks, 8 females and 3 males, attained maturity and were continued on experiment until approximately 16 months of age. Six of the surviving females and 2 of the males were markedly micromelic at the time of hatching. It was observed that the micromelic chicks at no time showed any apparent recovery from this condition.

Measurements of the shanks, the ulna sections of the wings and of the keels of the micromelic females were made at 11 months of age and compared with similar measurements of normal females of the same breeding and of approximately the same age and weight. No difference between the average lengths of the keels of these groups of hens was found but significant differences were revealed between the average lengths of the shanks and the average lengths of the ulna sections of the wings. A preliminary report of these results together with those on the ataxic condition has been made by Caskey and Norris.⁷

At the conclusion of the experiment the 6 micromelic females and 6 normal females of like breeding and of approximately the same age and weight were killed and their bones dissected and measured. The results of these measurements are given in Table I. A picture of one of the micromelic females is given in Fig. 1.

TABLE I.
Effect of an Embryonic Manganese Deficiency upon the Subsequent Bone Development of Chickens.

Bone measured	Length		Reduction in length, %	Significance of difference†
	Normal* hens, cm	Micromelic† hens, cm		
Sternum	13.70	13.60	0.7	—
Femur	9.14	8.15	10.8	332:1
Tibia	12.90	10.96	15.0	1110:1
Tarso-metatarsus	8.42	6.87	18.4	4999:1
Humerus	7.84	7.30	6.9	124:1
Radius	7.08	6.30	11.0	587:1
Ulna	7.92	7.17	9.5	768:1
Metacarpus	4.24	3.90	8.0	8:1

*N.H. x R.I.R., avg age 17 mo and avg wt 2034 g.

†N.H. x R.I.R., avg age 16 mo and avg wt 2032 g.

‡Odds as determined by "Student's" method (Z test).



FIG. 1.
A typical micromelic hen.

The average length of the sterna of the micromelic females was only 0.7% less than that of the normal females. The difference in length was obviously of no significance. The difference between the average length of the metacarpi of the micromelic females and that of the normal females amounted to 8%, but it did not prove to be statistically significant when analyzed by "Student's" method (Z test). All the other differences in bone length proved to be highly significant statistically. The tibiae and the tarso-metatarsi showed the most marked retardation in development, the tibiae being 15% shorter than the normal bones and the tarso-metatarsi 18.4% shorter. The shortening was somewhat greater than that previously reported by Caskey, Gallup and Norris⁴ in newly hatched chicks, but not as great as that reported by Landauer² or that reported by Lyons and Insko⁸ for 21-day embryos and newly hatched chicks.

It is evident from these results that newly hatched chicks which become micromelic during embryonic development as a result of manganese deficiency do not recover from the micromelia when fed a diet containing an adequate amount of manganese during a period greatly in excess of that required for the attainment of maturity. No evidence was obtained, on the other hand, of a reduction in the

length of the sternum as a consequence of embryonic manganese deficiency. The difference in the effect of the embryonic manganese deficiency upon the bones of the legs and wings and upon the sternum may be related to the fact that the former undergo considerable calcification during the latter stages of embryonic development whereas the sternum is almost entirely uncalcified at the time of hatching.

Byerly and associates¹ reported that the anterior-posterior axis of the skulls of the micromelic chicken embryos which they examined was markedly shortened. Both this group of investigators and Landauer² observed that some of the affected embryos hatched but Landauer² stated that the head was always normal. It has been observed at this laboratory, however, that some of the newly hatched chicks rendered micromelic by manganese deficiency during embryonic development also possessed brachycephalic heads. Several of the micromelic females which were sacrificed at 16 months of age in order to study the reduction in bone length were still brachycephalic. This indicates that similar to the micromelia due to manganese deficiency the consumption of a diet adequate in manganese after hatching does not promote recovery from the brachycephalism.

Summary. Chicks which are rendered micromelic during embryonic development as a result of manganese deficiency do not recover from this condition when fed a diet adequate in manganese during a period of time greatly in excess of that required for the attainment of maturity.

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Rôle of Vitamin C in Addison's Disease.*

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The excretion of ascorbic acid in the urine of patients having Addison's disease has been studied by Siwe,¹ von Drigalski,² Geriola,³ Wilkinson and Ashford.⁴ Using the method of Harris and Ray,⁵ all the aforementioned investigators found a state of vitamin C

* Abridgement of thesis submitted by Dr. Jenovese to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

deficiency in the cases studied. Wilkinson and Ashford concluded that not only was there a definite deficiency in vitamin C associated with cases of Addison's disease, but that a parallelism existed between the degree of deficiency and the severity of the disease. Sendroy and Miller⁶ made studies on the combined clearances of urea and ascorbic acid of 8 patients having nephritis and concluded that a relationship existed between the renal efficiency as indicated by the clearance of urea and the amount of ascorbic acid excreted. They suggested that in the presence of Addison's disease in which a functional renal insufficiency exists, the deficiency in the excretion of vitamin C is caused by the renal insufficiency rather than by a state of vitamin C subnutrition. In addition they found a parallelism between the values of urea clearance and the amount of vitamin C excreted in the urine.

The present study was undertaken to investigate further the rôle of vitamin C in Addison's disease by correlating the ascorbic acid content of the blood with the urinary excretion of vitamin C in 6 patients having Addison's disease. The studies on urinary excretion were made according to the method of Harris and Ray, utilizing a test dose of 500 mg of ascorbic acid. The ascorbic acid content in the blood plasma was determined simultaneously with studies of the urine. The blood urea was determined in every instance and, in 4 of the cases, studies of the clearance of urea were made. The investigations were carried out when the patients were first seen and the results reported herein represent the state of vitamin C nutrition that the patients had when they presented themselves for diagnosis. Owing to the fact that the patients were able to stay only a short time, it was possible to carry out saturation studies in only one instance.

Procedure. The subjects were 6 patients with Addison's disease and 6 normal persons as controls. One additional subject with a history of having ingested a diet low in vitamin C for 5 months was also studied. Essentially, the plan of study in each case was to determine first the content of ascorbic acid in the blood plasma in the fasting state. Then the 24-hour urinary excretion of ascorbic acid was determined. To determine the states of vitamin C saturation, the subjects received a test dose of 500 mg of ascorbic acid and the

¹ Siwe, Sture, *Klin. Wehnschr.*, 1935, **14**, 1311.

² von Drigalski, Wolf, *Klin. Wehnschr.*, 1935, **14**, 338.

³ Geriola, F., *Minerva med.*, 1937, **2**, 642.

⁴ Wilkinson, J. F., and Ashford, C. A., *Lancet*, 1936, **2**, 967.

⁵ Harris, L. J., and Ray, S. N., *Lancet*, 1935, **1**, 71.

⁶ Sendroy, Julius, Jr., and Miller, B. F., *J. Clin. Invest.*, 1939, **18**, 135.

TABLE I.
Vitamin C in Blood Plasma and Urine of 6 Normal (Control) Persons; Responses to Test Dose of Ascorbic Acid.

Control	Date	Vitamin C in mg per 100 ml	Vitamin C 24-hr urine, mg	Vol., 24-hr urine, ml	Vitamin C in 3-hr urine, mg, after 500 mg test dose ascorbic acid	Vol., 3-hr urine, ml
1	2-3-39	0.69	18.5	1600	3.6	215
2	1-20	1.15	35.4	800	57.1	110
3	1-21	1.48	58.2	1100	32.2	410
4	1-20	1.31	30.4	1500	57.1	
5	1-21	0.57	23.8	1210	13.3	350
6	1-23	0.97	24.0	1850	5.8	550

effect on the 3-hour urinary excretion of vitamin C was observed. The blood urea was determined in all cases.

The persons used as normal controls were all young physicians. They gave a history of having been free from recent acute infections or gastrointestinal disorders. The results in each case are contained in Table I.

Methods of Analysis. The method used for the determination of ascorbic acid in the urine was the technic recommended by Harris and Ray; ascorbic acid in the blood was determined by the method of Taylor, Chase and Faulkner.⁷ Both of these procedures were described in detail by Magnusson and Osterberg.⁸

Analysis of Results in Controls. The content of ascorbic acid in the blood plasma of the normal persons used as controls ranged from 0.57 to 1.48 mg per 100 ml of plasma. These values are in the normal range as reported by other workers. Similarly, the amounts of ascorbic acid excreted in the urine of the same (normal) persons in 24 hours were normal, varying from 18.5 mg to 58.2 mg (Table I).

The administration of a test dose of 500 mg of ascorbic acid resulted in a 3-hour urinary excretion ranging from 3.6 to 57.1 mg. Two subjects (1 and 6) gave results far below the normal response to be expected from this test (Table I). The other 4 subjects responded normally. Harris and Ray found that the excretion of ascorbic acid in the urine following a test dose of 500 mg of ascorbic acid was usually 8 to 10 times the normal excretion in a 3-hour period.

Experimental Data. Of the 6 patients who had Addison's disease, the patient in case 1 yielded entirely normal results (Table II). In

⁷ Taylor, S. H. L., Chase, Dorrance, and Faulkner, J. M., *Biochem. J.*, 1936, **30**, 1119.

⁸ Magnusson, Arlene E., and Osterberg, A. E., *Proc. Staff Meet., Mayo Clin.*, 1938, **13**, 700.

TABLE II.
Data from 6 Cases of Addison's Disease.

Case	Vitamin C in plasma, mg per 100 Ml	Vitamin C 24-hr urine, mg	Vol., 24-hr urine, Ml	Vitamin C in 3-hr urine, mg, after 500 mg test dose ascorbic acid	Vol., 3-hr urine, Ml
1	1.57	20.1	2420	103.50	600
2	0.95	10.4	2730	1.34	365
3	1.22	5.01	1000	33.70	100
4	0.71	13.1	625	2.02	148
5	0.94	10.0	1100	4.40	417
6	1.15	7.84	1290	4.4	100

this case the history of Addison's disease was of short duration. In addition, there was a history of a more than adequate daily intake of vitamin C in the form of a pint or more of tomato juice.

In the other 5 cases studied (Table II) the ascorbic acid in the blood plasma was also normal or high normal. However, in these cases the amounts of ascorbic acid excreted by the patients in the urine during 24 hours were definitely lower than normal. The values ranged from 5.01 mg to 13.1 mg. According to our normal control subjects and the results reported by others, the normal range of the urinary excretion of ascorbic acid per 24 hours is between 20 and 30 mg. Wilkinson and Ashford reported this same observation on the urinary excretion of ascorbic acid in the cases of Addison's disease that they studied.

The patient in case 6 (Table III) was observed over a longer period than were the other patients. The daily urinary excretion of ascorbic acid of this patient remained low until 500 mg of ascorbic acid was administered intravenously. The excretion value following this procedure increased to 26 mg per 24 hours. Three days after the initial intravenous dose of ascorbic acid had been administered, 500 mg of ascorbic acid was given orally and 500 mg was administered intravenously. The next 3-hour urine specimen contained 195.7 mg of ascorbic acid and during the following 21 hours 448.7 mg was excreted, making a total excretion of 644.4 mg for the 24-hour period, or approximately 65% of the amount administered to the patient. It would seem that the tissues of this patient must have been saturated with vitamin C to permit such an excretion in 24 hours, yet 5 days after this observation the 24-hour specimen of urine of this patient contained only 16 mg of ascorbic acid. The blood plasma the next day contained 2.01 mg of ascorbic acid, a value which is decidedly above the threshold value of 1.4 mg reported in the literature.

TABLE III.
Data from Detailed Study of Case 6.

Date	Vitamin C in plasma, mg per 100 Ml	Vitamin C in 24-hr urine, mg	Vol., 24-hr urine, Ml	Vitamin C in 3-hr urine, mg, after 500 mg test dose ascorbic acid	Vol., 3-hr urine, Ml
12-30-38	1.15	7.84	1290		
1- 6-39		11.5	1125		
1- 9		11.1	1175		
1-10	1.05				
1-11		11.47	1425		
1-12		15.2	1100		
1-13		9.7	975		
1-13				5.2	100
1-15		8.9	1050		
1-16		5.4	925		
1-16 (500 mg ascorbic acid intravenously)				181.0	150
1-18		26.0	950	31.7	125
1-19 (500 mg ascorbic acid intravenously; 500 mg ascorbic acid orally)				195.7	1125
1-20 (21-hr specimen urine contained 448.7 mg in volume of 1700 Ml)				63.4	134
1-21				22.4	50
1-26		16.0	800		
1-27	2.01				
1-30		23.0	1300		
1-31		8.9	1200		
2- 1		24.7	1375		

The observations made in this particular case (Table III) would suggest that perhaps the urinary excretion of vitamin C alone cannot be taken as an adequate criterion for the diagnosis of vitamin C subnutrition. The blood plasma value of the vitamin was normal in all cases studied and yet studies of urinary excretion revealed a low excretion of the vitamin.

Summary and Conclusions. The blood plasma values for ascorbic acid in the 6 patients who had Addison's disease that were studied were within normal range. The urinary excretion of ascorbic acid during a 24-hour period was low. On the basis of the results obtained, it would seem that the urinary excretion of ascorbic acid alone cannot be used as an index of vitamin C deficiency in instances of Addison's disease.

Effects of Salts and Adrenal Cortical Extracts upon Toxicity of Drugs.

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Verzàr and his coworkers¹ in discussing the possible relationships between the adrenal cortex and fat, carbohydrate, and electrolyte metabolism, have postulated that the adrenal cortex controls a wide number of metabolic processes by regulating phosphorylation processes.

One of the primary arguments of these workers is that the effects of iodoacetate poisoning seemed to reproduce some of the symptoms of adrenal insufficiency, such as impaired selective intestinal absorption of glucose, muscular asthenia, steatorrhea, lowered body temperature, fluid loss by diarrhea, etc. These effects are ascribed to a specific inhibitory action of iodoacetate upon phosphorylation.

Laszt² has found that NaCl therapy antagonizes the iodoacetate effects in the intact rat, with respect to fatal toxicity, and impaired intestinal glucose absorption. This observation was offered as a possible explanation of some of the beneficial effects of NaCl therapy in adrenal insufficiency.

It appeared possible that this so-called "experimental adrenal insufficiency" produced by iodoacetate, could be explained on another basis than by specific inhibition of phosphorylations. This possibility was brought to our attention by the well known efficacy of NaCl, base, and fluid therapy in many toxic conditions, such as in mercury poisoning,³ in which fluids are lost by emesis and diarrhea, and uremia ensues as a result of kidney damage. Such toxic agents could not be expected specifically to inhibit phosphorylations under the control of the adrenal cortex, although some of them may produce changes which have been termed symptoms of the "alarm reaction" by Selye and his coworkers.⁴ For this reason, we have investigated the

¹ Verzàr, F., *Die Funktion der Nebennierenrinde*, Basel, Benno Schwabe & Co., 1939; *Absorption from the Intestine*, Longmans, Green & Co., N. Y., 1936.

² Laszt, L., *Nature*, 1939, **144**, 244.

³ Haskell, C. C., Carder, J. R., and Coffindaffer, R. S., *J. Am. Med. Assn.*, 1923, **81**, 448.

⁴ Selye, H., *Archiv. Internat. Pharm. Theràp.*, 1937, **55**, 431; *Am. J. Physiol.*, 1938, **123**, 758.

effects of NaCl, KCl, fluids, and adrenal cortical hormone upon rats poisoned with several non-specific toxic agents, including iodoacetate.

Four groups of 10 rats each were divided into 2 subgroups averaging ca 150 g per rat, each group receiving subcutaneously 50 and 80 mg per kg of sodium iodoacetate, respectively. This dosage is distinctly a lower level than the 100-120 mg level employed by Laszt. The rats were fasted and placed on the following *ad libitum* drinking fluids: (1) tap water, (2) 0.6% NaCl, 0.2% Na Citrate, (3) 0.2% KCl, and (4) tap water. Each group was given by stomach-tube 1 cc per sq decimeter of body surface of the following fluids: (1) tap water, (2) 1% NaCl, 0.1 N NaHCO_3 (ratio of 3:1) or 0.6% NaCl, 0.2% Na Citrate, (3) 1% KCl, and (4) tap water. The latter group was injected subcutaneously with Upjohn adrenal cortical extract, assaying 2.5 Cartland-Kuizenga rat units⁵ per cc, at a level of 1 cc per rat at the time of administering iodoacetate, and again 4 hr later. The stomach-tubed fluids were given 4 hours before iodoacetate, at the same time as iodoacetate, and every four hours thereafter. Rectal temperatures were taken on all animals every 4 hours, and were seen to drop to approximately 95°F 4 hours after iodoacetate. Eight hours after the 50 mg level of iodoacetate, the NaCl group had nearly regained normal body temperature, with complete return in 12 hours; while all the other groups were still below 96°F at this time. All rats displayed muscular weakness progressing to collapse during the height of the effects of iodoacetate, and marked edema and inflammation at the site of injection, hemorrhagic diarrhea and hematuria. The NaCl group had a marked thirst for the NaCl-Na Citrate drinking fluid, and after 12 hours of fasting, the group which received the 80 mg level of iodoacetate had gained 37 g, showing a generalized edema.

The groups which received the 80 mg level all died within 8 hours except the NaCl group which survived, with return from 95° temperature to 98° within 20 hours. Potassium chloride decreased survival time, while cortin had no beneficial effects as compared with the controls on tap water. Another experiment, using 12 rats, was performed, in which survival times of cortin-treated and control iodoacetate-poisoned rats were compared for survival. More cortical extract was used than in the previous experiment (Wilson extract, assaying 1 d'Armour unit per 0.1 cc, 0.2 cc per 100 g body weight. Rats weighed av. 325 g), and was given at the time of administration of iodoacetate, and again 6 hours later. No effects

⁵ Cartland, G. F., and Kuizenga, M. H., *J. Biol. Chem.*, 1936, **116**, 57.

on survival were obtained, thus confirming the previous result that cortin seems to have no beneficial effect on iodoacetate-poisoning.

Colchicine has been used by Leblond and Sagal⁶ in a study of Selye's alarm reaction in rats. There are, however, no reasons to believe that this drug is a specific inhibitor of phosphorylations.

Preliminary experiments indicated that the minimal lethal dose of subcutaneously administered colchicine is less than 1 mg per kg, death being delayed. Food was offered *ad libitum* in the experiments with colchicine. Pathological symptoms were: collapse, weakness, lowered body temperature, hemorrhagic diarrhea and hematuria, loss of muscular tone, and poor muscular control.

Two levels of colchicine were given, 1 and 2 mg per kg, and essentially the same types of salt and fluid medication as described for the iodoacetate experiments. On the 2 mg level, in contrast to the iodoacetate results, KCl and cortin, as well as NaCl, exerted a beneficial effect as compared with the controls, with respect to body temperature, general activity, and survival. NaCl and cortin, especially the latter, exerted more marked effects. Fig. 1 illustrates the effect of NaCl and cortin on body temperature. It is noticed that the NaCl and especially the cortin groups nearly regain normal

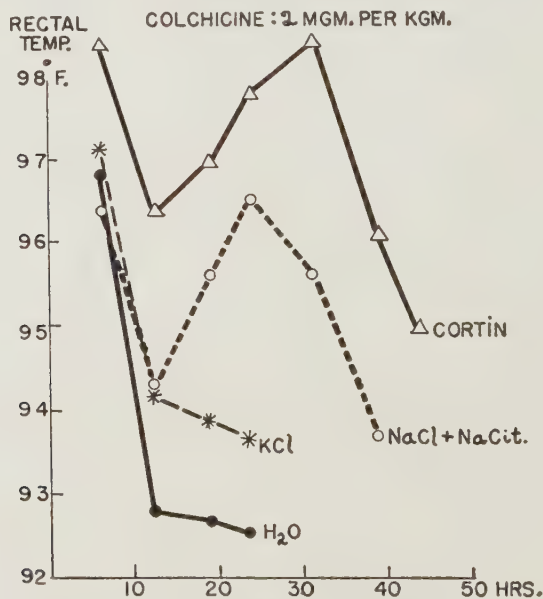


FIG. 1.
Effect of H₂O, NaCl-citrate, KCl, and Cortin Administration on Body Temperatures of Colchicine-Poisoned Rats.

⁶ Leblond, C. P., and Segal, G., *Compt. Rend. Soc. Biol., Paris*, 1938, **128**, 995.

temperatures, although the effects were not permanent at this level of colchicine. At the lower level of colchicine, the same results were obtained, except that several of the NaCl, KCl, and cortin animals survived indefinitely, whereas all the controls died.

Preliminary results demonstrated a minimal lethal dose of 10 mg per kg of subcutaneously administered HgCl_2 in rats. Death was markedly delayed, hence food was offered. Four groups of rats, averaging ca 240 g, were injected with 20 mg per kg of HgCl_2 , thus a lethal dose. The groups were treated essentially as in the previous experiments, except that the hormone was given as subcutaneously administered desoxycorticosterone acetate in oil ("Doca") instead of cortical extract. One mg in oil was injected 0, 12, 24, 34, 43, 43 hr, and 2 mg at 51, 58, and 68 hr. The stomach-tubed fluids were administered at 24, 30, 35, 45, 60, 69, 80, 92, 104, 164, and 192 hr. Potassium chloride was definitely toxic to HgCl_2 poisoned rats, while "Doca" had no effect. The general symptoms were quite similar to those in iodoacetate poisoning except that death was delayed. All but one of the NaCl group had nearly regained normal weight within 2 weeks, and survived indefinitely. All others died; the KCl group first, followed by the "Doca" group, then the controls.

In summary, Fig. 2 shows the pooled results, with respect to survival times of rats poisoned with all 3 toxic agents. The top row of figures shows indefinite survival in all groups on the 50 mg level of

TOXIC AGENT & MGM. PER KGM	MEDICATION AND AV. HRS. SURVIVAL								MLD. MGM. PER KGM.
	NO. RATS	H_2O	NO. RATS	NaCl , NaCit.	NO. RATS	KCl	NO. RATS	CORTIN	
IAA 50	5	∞	5	∞^*	5	∞	5	∞	>50 <80
IAA 80	11	8	5	∞^*	5	5	8	7	
COLCH. 1	4	62	4	2- ∞ 2-75	4	2- ∞ 2-66	4	1- ∞ 3-75	<1
COLCH. 2	7	20	4	28	4	30	4	43	
HgCl_2 20	13	89	7	6- ∞ 1-144	7	43	7	79 [✱]	10

* $\text{NaCl} + \text{NaHCO}_3$

✱ DESOXYCORTICOSTERONE ACETATE

FIG. 2.

Effect of H_2O , NaCl, KCl, and Cortin Administration on Survival of Iodoacetic-, Colchicine-, and HgCl_2 -Poisoned Rats.

iodoacetate. The second row shows the marked life-maintaining effect of NaCl therapy in the case of lethal doses of iodoacetate, and lack of effect of cortin, while KCl seems somewhat toxic. The fourth row illustrates the beneficial effects of cortin in fatal colchicine poisoning. The last row of figures illustrates the toxicity of KCl, benefits of NaCl, and lack of effects of "Doca" in mercury poisoning. Temperature trends showed the same results as survival data, and in some cases revealed beneficial or detrimental effects much better throughout the entire study, except in the case of mercury poisoning.

Shorr, Barker, and Malam⁷ questioned the specific inhibition of phosphorylation by iodoacetate during glucose oxidation. Wertheimer,⁸ Klinghoffer,⁹ Öhnell and Höber,¹⁰ and Doty and Eaton,¹¹ have stated that iodoacetic acid has no effect upon intestinal absorption of sugar, salts, and amino acids by any specific inhibitory action, but does so if present in grossly pathological quantities, in which the toxic symptoms described in this paper would result. Most workers have used much larger doses than we have employed. Verzàr and his coworkers have used impaired intestinal glucose absorption as a criterion of adrenal insufficiency, and have extended reasoning obtained in such experiments to other experiments in which phosphorylation in general is claimed to become impaired in adrenalectomized animals. The data we have presented in addition to the quoted references seem to us to constitute an argument against comparing the non-specific pathology of iodoacetate poisoning with the symptoms seen in the adrenal insufficiency syndrome.

⁷ Shorr, E., Barker, S. B., and Malam, M., *Science*, 1938, **87**, 168.

⁸ Wertheimer, E., *Archiv. ges. Physiol.*, 1933, **233**, 514.

⁹ Klinghoffer, K. A., *J. Biol. Chem.*, 1938, **126**, 201.

¹⁰ Öhnell, R., and Höber, R., *J. Cell. and Comp. Physiol.*, 1939, **13**, 161.

¹¹ Doty, J. R., and Eaton, A. G., 52nd Proc. Am. Physiol. Soc., 1940, p. 50.

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Transfer of Radioactive Sodium Across the Placenta of the Cat.*

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Radioactive sodium, Na_{24} , was prepared by use of the electrostatic pressure generator of the Department of Terrestrial Magnetism, Carnegie Institute of Washington. Samples emitting about 10^5 beta-rays per second were injected intravenously, as the chloride, into pregnant cats. At various intervals of time after injection, fetuses were removed by Caesarian section and a sample of blood taken from the mother. The radioactivity of the samples, in terms of beta-particles per second, was measured by a pressure ionization chamber and string electrometer, using the method previously described.¹

Typical data are presented in Tables I and II. Analysis of these data reveal the following relationships:

1. Table I. The fetus near term comes to within 10% of a limiting equilibrium value with respect to sodium ion in the maternal plasma only after 12 to 18 hours. This is in striking contrast to the extracellular fluid of the mother which comes to the same value in about 4 minutes.²

2. Table II, column 6. The rate of transfer across the placenta per unit weight of placenta is very low in early stages of pregnancy (gestation age, 15 to 20 days) but increases in linear manner to a

TABLE I.

Delivery time of fetus after injection of Na_{24} , hours	Fetal wt, g	Betas/sec/ total fetus*	Betas/sec/ g fetus
1.0	132	2.11	.0160
6.7	116	11.9	.102
19.0	96	19.5	.206
23.5	130	26.0	.200

*In each instance the number of beta-particles per second emitted by the sample (in all directions) has been corrected for background and radioactive decay and to unit activity of the maternal blood plasma. This makes the data from the several experiments immediately comparable.

These fetuses have a gestation age of 55 days or over.

* Aided by a grant from the Rockefeller Foundation Fluid Research Fund of the School of Medicine, Johns Hopkins University.

¹ Flexner, L. B., and Roberts, R. B., *Am. J. Physiol.*, 1939, **128**, 154.

² Hevesy, G., *J. Chem. Soc.*, 1939, **1939**, 1213.

value 60 times that of the early stage at a gestation age of 57 days. After this stage, the rate of transfer per unit weight of placenta decreases somewhat until term (62 days).

3. Table II, column 5. The rate of transfer to a unit weight of fetus, however, is high in early stages and falls with the duration of pregnancy. For example, the ratios of rates of transfer for gestation ages of 15-20 days, 40 days and 60 days are 5.5:2.5:1. The relatively high rate of transfer per unit weight of fetus in the youngest fetuses is accounted for by the large size of the placenta compared to that of the fetus (Table II, columns 2 and 3).

TABLE II.

Gestation age days	Fetal wt, g	Placental wt, g	Betas/sec/ total fetus/hr*	Betas/sec/g fetus/hr	Betas/sec transferred/g placenta/hr
15-20	.15	7	0.013	.085	.002
40	15.0	17.7	0.61	.041	.035
50	52.0	13.5	1.20	.023	.089
57	102.0	13.6	1.60	.0157	.118
62	132.0	25.4	2.11	.0160	.083

*Corrected for background and radioactive decay, and to unit activity of the maternal blood plasma as in Table I.

4. Using the data of Coronios,³ a curve has been constructed relating the percentage daily increase in fetal weight to the fetal age. This curve parallels a curve relating the rate of transfer of Na_{24} per unit weight of fetus to fetal age. It consequently appears that changes in rate of fetal growth in the cat are accompanied by parallel changes in rate of placental transfer per unit weight of fetus.

The study presented here is part of a comprehensive investigation now proceeding on the comparative physiology of the placenta as revealed by radioactive isotopes.

We are indebted to Dean B. Cowie of the National Cancer Institute for making the sodium bombardments with the Carnegie generator.

³ Coronios, J. D., *Genetic Psychol. Monographs*, 1933, **14**, 283.

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Specific Nature of Complement Fixing Antibody in Malaria as Demonstrated by Absorption Tests.

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(Introduced by O. S. Gibbs.)

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We¹ have confirmed the demonstration by Coggeshall and Eaton^{2,3} of a specific complement fixation reaction in malaria employing *P. knowlesi* antigens. The parasites were washed as free as possible of hemoglobin and other blood constituents and dried *in vacuo*. When ready for use a standardized amount was rehydrated with physiological saline, frozen and thawed, and the supernatant fluid used as antigen.

We have tested sera from 83 patients in whose blood malaria parasites were demonstrated. Seventy-two percent gave a positive complement fixation for malaria at some time during the course of the disease. The positive reaction was correlated with the presence or recent presence of demonstrable parasites but not with the number of parasites. Our results show that a positive complement fixation reaction with our parasite antigen is probably diagnostic of malaria. However, a negative reaction does not rule out malaria.

Sera from 134 individuals presumably free from malaria yielded 127 negative and 7 weakly positive reactions read as 1+ or \pm . Forty-three of these sera were known to give a positive Wassermann reaction, 40, a negative Wassermann. One in each group gave a weakly positive reaction with the malaria antigen. It would appear that syphilis does not provoke non-specific reactions with the malaria parasite antigen.

We have further shown that Wassermann negative patients who received induced malaria remained Wassermann negative throughout the course of their treatment, even after they had developed a strongly positive reactivity for the malaria antigen.

Absorption experiments also indicate distinct and unrelated antibodies since treatment of serum with either the malaria or Wassermann antigen removes the specifically reacting substance from that

¹ Stratman-Thomas, Warren K., and Dulaney, Anna Dean, *Am. J. Trop. Med.*, in press.

² Eagle, Harry, and Hogan, Ralph B., *J. Exp. Med.*, 1940, **71**, 215.

³ Coggeshall, Lowell T., and Eaton, Monroe D., *J. Exp. Med.*, 1938, **67**, 871.

serum without modification of its ability to fix complement in the presence of the other antigen.

For these absorption experiments sera giving 4+ reaction with both the malaria and Wassermann antigens were used. We followed the procedure employed by Eagle² in absorption experiments on syphilitic sera using Wassermann and Reiter spirochetal antigens.

The 5 sera tested were obtained from patients who had received induced malaria therapy for the treatment of paresis. Sera obtained from these patients prior to the malaria inoculation gave 4+ Wassermann reactions and no complement fixation with the malaria antigen.

Each serum was divided into 5 portions. Part 1 (2 cc) was absorbed with the sediment from 2 cc of Kahn antigen which had been diluted with 3.5 cc of saline and centrifuged. After incubation for 2 hours at 37.5°C and overnight in the ice box 2 cc of physiological saline were added. Removal of the lipoidal particles was accomplished by centrifugation at high speed and subsequent filtration through a micro-Seitz filter. Part 2 (2 cc) was diluted with an equal volume of physiological saline and served as a control for the filtering process to which Part 1 was subjected. Part 3, (2 cc) was combined with an equal volume of the undiluted *P. knowlesi* antigen, prepared in our routine manner. After incubation for 2 hours at 37.5°, the mixture was centrifuged and the supernatant fluid removed. One cc (KAb₁) was set aside for testing and the remainder used for a second absorption with the undiluted antigen. In some cases this process was repeated again and the final mixture of serum and antigen left in the ice box overnight when it was centrifuged and the supernatant fluid (KAb₃) removed for testing. Part 4 (2 cc) was absorbed 2 or 3 times with an antigen prepared from red blood cells of normal monkeys to rule out species factors which might influence the reaction with the malaria antigen. Part 5 was used as the untreated serum control.

Complement fixation tests on non-absorbed and absorbed portions of the sera were done at the same time with Wassermann antigen and our *P. knowlesi* malaria antigen. The procedure employed for our routine complement fixation tests was followed. Serum and antigen controls were included. Table I gives the detailed results obtained with the serum of patient DIS. Other sera yielded similar data.

These results show: (1) Absorption with the Wassermann antigen removes the so-called syphilitic reagin, responsible for the positive Wassermann test without affecting the reactivity of the serum with

TABLE I.
Effect of Absorption with Wassermann, Malaria, and Normal Monkey Antigens on
Reactivity of Serum for Wassermann and Malaria Antigen.

Serum of patient dis.	Antigen	Dilutions of serum						
		Undiluted	1:2	1:4	1:8	1:16	1:32	1:64
Untreated	*	4+	4+	4+	4+	2+	—	—
	†	4+	4+	4+	1+	—	—	—
Diluted 1:2 with saline and filtered	*	—	4+	4+	3+	2+	—	—
	†	—	4+	3+	—	—	—	—
Absorbed with Kahn antigen, diluted 1:2 with saline, filtered	*	—	4+	4+	3+	1+	—	—
	†	—	—	—	—	—	—	—
Absorbed with malaria antigen 1 x (KAb ₁)	*	—	3+	2+	1+	—	—	—
	†	—	4+	4+	—	—	—	—
Absorbed with malaria antigen 2 x (KAb ₂)	*	—	—	1+	—	—	—	—
	†	—	—	4+	—	—	—	—
Absorbed with malaria antigen 3 x (KAb ₃)	*	—	—	—	—	—	—	—
	*	—	—	—	—	—	—	—
Absorbed with normal mon- key antigen 1 x (NAb ₁)	*	—	4+	4+	4+	2+	—	—
	†	—	4+	4+	±	—	—	—
Absorbed with normal mon- key antigen 2 x (NAb ₂)	*	—	—	4+	3+	2+	—	—
	†	—	—	4+	1+	—	—	—
Absorbed with normal mon- key antigen 3 x (NAb ₃)	*	—	—	—	3+	1+	—	—
	†	—	—	—	—	—	—	—

*Malaria.

†Wassermann.

the malaria antigen to a significant degree. (2) Absorption with the malaria antigen removes the malaria antibody without removal of the syphilitic reagin to a significant degree. (3) Absorption with normal monkey red cell "antigen" does not affect the reactivity of the serum with either the Wassermann or the malaria antigen. 4. On the basis of this investigation the complement fixation test for malaria would appear to be indicative of the presence of a specific malaria antibody in patients' sera. 5. The syphilitic reagin and the malaria antibody would appear to be distinct entities.

Functional Properties of Isolated Spinal Cord Grafts in Larval Amphibians.*

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The thesis that the operation of the central nervous system can be satisfactorily explained in terms of fixed neurone arrangements, rests largely on the interpretation of the structural organization of the central gray, as it presents itself in the normal animal. If the central nervous system really owes its fundamental functional manifestations to the minute details of its neurone architecture, any major disorganization of the latter should thoroughly derange the former. Accordingly, a study of the functional capacities of a central nervous system whose anatomical connections have been thrown into confusion promises information of crucial interest. Such a condition can be produced by transplantation.

Fragments of spinal cord, including several segments, excised from larval salamanders (*Amblystoma punctatum*) were grafted into the gelatinous connective tissue of the dorsal fin fold. Hosts and donors were of identical age (ca 2 cm in length) and had been in full functional activity for many weeks. In 7 of the 14 animals thus operated a limb was grafted at some distance anteriorly or posteriorly to the cord graft. All grafts became quickly vascularized and well incorporated.

Histological study revealed 3 main changes in the grafted cord fragments: (1) varying degrees of reduction of the gray matter; (2) considerable deformation and disorganization of the surviving portion; (3) outgrowth of bundles of nerve fibers into the surroundings.

The outgrowing nerve fibers form connections with skin, trunk muscles, and in the presence of a grafted limb, also with the latter. The cables connecting cord and limb grafts are always much stronger than other bundles. This fact, highly significant for the interpretation of normal nerve development, suggests that the first pioneering fibers to become attached to the limb thereby acquire some faculty—a kind of “stickiness”, as it were—converting them into a preferential contact pathway for later fibers. Arrived

* Aided by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of The University of Chicago.

inside the limb, the fibers form abundant and typical connections with muscles and skin.

Within a few weeks of the transplantation these isolated cord-limb complexes begin to exhibit functional activity, in which 3 successive phases can roughly be identified: an early phase of "spontaneous" activity; a later phase of responsivity to stimuli applied to the grafted center; a final phase of true reflex responsivity.

The first phase is characterized by intermittent or almost incessant twitching of the limb muscles. The twitches usually appear in spells, starting with irregular fibrillations and gradually building up to violent convulsions. A single fit may last for several minutes. At the peak of activity, the contractions are remarkably well synchronized, the limb executing strong periodic beats, sometimes at fairly regular intervals of the order of one to several seconds. The seizures appear no matter whether the animal is at rest or in motion, but are usually more intense following a period of host activity.

During the following weeks the spontaneous bursts become scarcer, with longer periods of inactivity separating the individual fits. During this phase reactions can, however, often be evoked by lightly pressing against the cord graft; the response follows the stimulus with a latency of sometimes more than a second and consists of anything from a single jerk to a seizure of several minutes' duration.

A few weeks later true reflex responses can usually be obtained by tactile stimulation (with cotton fibers) of the skin in the vicinity of the cord graft. In the course of time the stimulogenous area increases. The reflex response consists of a vigorous indiscriminate contraction of all limb muscles with no sign of coördination. The form of the response is essentially constant for a given case, but its size and temporal characteristics vary with the strength and mode of application of the stimulus, as well as with the condition of the host body. While a weak localized stimulus may yield a single twitch, an increase in the strength of the (mechanical) stimulus or spatial summation (stroking) or temporal summation (repetitive touch) all produce a repetitive response, with the after-discharge sometimes lasting for several seconds. Moreover, the excitability of the preparation fluctuates with the condition of the host body: prolonged host activity is invariably followed by a marked increase in the reflex excitability of the cord graft. This host influence on graft excitability is a humoral effect, since direct nerve connections between the central nervous system of the host and the cord graft are lacking.

In cases in which the grafted cord had innervated trunk muscles, the latter showed reactions similar to the ones described for the limb grafts. The contractions of the segmental muscles were always directed towards the site of the cord graft as the center of innervation. As in the limb cases, irregular fibrillations as well as synchronized beats were observed; in a few instances, slow, tonic, contractions were also noted.

The reactions described in the preceding are positively neurogenic manifestations of the isolated cord-limb graft complex itself. As a crucial check against the possible intrusion of host innervation, the host cord was pithed in several specimens, and finally the portion of the back containing the grafted units was completely excised and tested in isolation. Even so, the preparations exhibited the same functional activities as before. In fact, their excitability was even markedly increased.

These observations demonstrate that a fragment of spinal cord, after undergoing a major involution, is still in possession of certain functional properties which, accordingly, can be regarded as the fundamental dynamic properties of a nerve center deprived of its finer structural differentiation. Thus far the following have been observed in our preparations: Spontaneous firing (later subsiding); long latency; synchronization of discharges; after-discharge; repetitive action; reflexivity; spatial summation; temporal summation; fluctuating excitability; fatigue.

The details of the microscopical examination of the grafts will be reported later. The most surprising fact is the presence of abundant sensory fibers, serving as afferent pathways in the described reflexes, despite the fact that only pure spinal cord without primary afferent neurones had been transplanted. Apparently, fiber processes of the spinal gray have connected directly with the skin and become afferent in function, with a concomitant reversal of the sense of their synaptic transmission. The structural disorganization of the gray has presumably weakened the original polarity and irreciprocity of synaptic relations.

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Differentiation of Sera of Two Species of Doves and Their Hybrid.*

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Following the studies of Nuttall,¹ the precipitin-reaction has come into wide use as a means of correlating the taxonomic relationships of species with the serological properties of their serum-proteins. For papers citing the numerous reports of work of this nature, the reader is referred to other sources.²⁻⁶ A few workers have employed the method of absorption of precipitins to differentiate in precipitin reactions, the serum-proteins of species that are distinguishable either not at all or with difficulty by direct precipitation. These are summarized by Cumley.⁷ (Landsteiner and van der Scheer⁸ have pointed out limitations in the principles of absorption of precipitins, which affect the interpretation of the results obtained by this technic.) We are familiar with only a few reports⁹⁻¹¹ dealing with the antigenic relationships of the serum of a species-hybrid to that of each of the parents. In the present tests, the serums of the Pearlneck (*Streptopelia chinensis*), Ring dove (*St. risoria*) and their hybrids (F₁-P.N./R.D.) were compared.

The precipitins were produced by injecting 0.6 cc of the serum of a species into rabbits 3 times per week until 6 cc of serum had been injected. The immune sera were collected on the tenth and eleventh days after the last injection. For absorptions, the undiluted anti-

* Paper No. 259 from the Department of Genetics, University of Wisconsin. This investigation was supported in part by a grant from The Rockefeller Foundation.

¹ Nuttall, G. H. F., *Blood Immunity and Blood Relationship*, 1904, Cambridge, The University Press.

² Baier, J. C., Jr., *Physiol. Zool.*, 1933, **6**, 91.

³ Boyden, A., *Am. Nat.*, 1934, **68**, 516; *Sigma Xi Quart.*, 1936, **24**, 152.

⁴ Hicks, R. A., and Little, C. C., *Genetics*, 1931, **16**, 397.

⁵ Hektoen, L., and Cole, A. G., *J. Inf. Dis.*, 1932, **49**, 29.

⁶ Wolfe, H. R., *Biol. Bull.*, 1939, **1**, 108; *Zoologica*, 1939, **24**, 309.

⁷ Cumley, R. W., *Am. Nat.*, 1939, **73**, 375.

⁸ Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1924, **40**, 91.

⁹ Ishihara, M., and Misao, T., *Jap. J. Gen.*, 1929, **4**, 147.

¹⁰ Kraus, R., and Prizbram, H., *Zentralbl. f. Physiol.*, 1907, **21**, 258.

¹¹ Sasaki, K., *Jap. J. Zootechn. Sci.*, 1926, **2**, 1; *Z. f. Tierzuchtung und Zuchtungsbiol.* (B), 1937, **38**, 361.

serum to one species was mixed with an equal volume of the serum of the other species, and stored at 2-5°C for 24 hours. The process was repeated, using smaller volumes of the absorbing serum, if, after centrifugation of the mixture, the supernatant fluid produced a ring when tested with this serum. For the precipitin-tests, the antiserum was placed in each of a series of capillary tubes of about 2 mm diameter, to a height of approximately 2 mm, and the antigen in its successive dilutions was carefully layered above. The appearance of a ring at the interface within 2 hours was taken as indication of a precipitate.

TABLE I.
Results of Ring-Precipitin Tests, with Anti-Pearlneck and Anti-Ring Dove Sera.

Antiserum No.	Antiserum	Absorbed by serum of	Antigens tested	Highest dilution of antigen giving a precipitate
39S3	Pearlneck	—	Pearlneck	1:16,384
"	"		Ring dove	1:16,384
"	"		F ₁ P.N./R.D.	1:16,384
39S3	"	Ring dove	Pearlneck	1:1024
"	"	"	Ring dove	None
"	"	"	F ₁ P.N./R.D.	1:32
39S3	"	F ₁ P.N./R.D.	Pearlneck	None
"	"	"	Ring dove	None
"	"	"	F ₁ P.N./R.D.	None
198S3	"	—	Pearlneck	1:32,768
"	"		Ring dove	1:32,768
"	"		F ₁ P.N./R.D.	1:32,768
198S3	"	Ring dove	Pearlneck	1:2048
"	"	"	Ring dove	None
"	"	"	F ₁ P.N./R.D.	1:64
198S3	"	F ₁ P.N./R.D.	Pearlneck	None
"	"	"	Ring dove	None
"	"	"	F ₁ P.N./R.D.	None
263S1	Ring dove	—	Pearlneck	1:16,384
"	"		Ring dove	1:16,384
"	"		F ₁ P.N./R.D.	1:16,384
263S1	"	Pearlneck	Pearlneck	None
"	"	"	Ring dove	1:512
"	"	"	F ₁ P.N./R.D.	1:128
263S1	"	F ₁ P.N./R.D.	Pearlneck	None
"	"	"	Ring dove	None
"	"	"	F ₁ P.N./R.D.	None
287S2	"	—	Pearlneck	1:16,384
"	"		Ring dove	1:16,384
"	"		F ₁ P.N./R.D.	1:16,384
287S2	"	Pearlneck	Pearlneck	None
"	"	"	Ring dove	1:1024
"	"	"	F ₁ P.N./R.D.	1:128
287S2	"	F ₁ P.N./R.D.	Pearlneck	None
"	"	"	Ring dove	None
"	"	"	F ₁ P.N./R.D.	None

Concentrated antiserum was used in the tests if the antisera were not absorbed. In the absorption-tests, the antisera were diluted in varying degrees, depending upon the amount of antigen required for absorption.

The results of the tests involving anti-Pearlneck and anti-Ring dove sera, either unabsorbed or following absorption by the serum of the other species or the hybrid, with the various dilutions of the sera of Pearlneck, Ring dove and their F_1 hybrid, respectively, are given in the table. It will be noted that the sera of Pearlneck, Ring dove, and their F_1 , respectively, reacted at the same dilution with each of the various unabsorbed antisera, and that no distinction between them could be made by these tests.

However, when anti-Pearlneck serum was absorbed by Ring dove serum, the reagent thus produced precipitated the serum of both Pearlneck and the F_1 , but not that of Ring dove. Likewise, following absorption of Ring dove antiserum by the serum of Pearlneck, the test fluid reacted with the serum of Ring dove and the hybrid, but not of Pearlneck. The serum of the species-hybrid invariably showed a precipitate at a lower dilution with the absorbed fluids than did the serum of either Pearlneck or Ring dove with their respective homologous antisera. Therefore, we may reasonably conclude that the serum of the species-hybrid contains a part, possibly all, of the same, or of related proteins that make for the species-specific qualities of the sera of each of the parental species. Additional evidence as to the possible relationship of the serum-proteins of the hybrids with those of each parent is furnished by the reactivity of the antisera for either parent following the respective absorptions by the hybrid serum. As will be noted, such reagents produced no precipitates at all with any of the 3 kinds of serum. Thus, since the hybrid serum by absorption could remove all the precipitins from the antiserum for either parent, it would seem that the antigenic components of the serum of this species-hybrid were very similar to, if not identical with, those of the serum of both parental species.

Investigations are now under way to determine the reactivity of the serum of backcross individuals (to Ring dove), representing the different unit-cellular characters of Pearlneck.¹²

Summary. The serum proteins of two dove species, Pearlneck and Ring dove, and their hybrid were indistinguishable by direct precipitin-tests; *i.e.* the three kinds of serum reacted at the same dilution with antisera for each of the two species. Following absorption, however, of the antiserum to one species by the serum of the other, a differentiation of the serum-proteins was readily made. The serum of the species-hybrid appeared to possess a combination of the precipitinogens of both parental species.

¹² Irwin, M. R., *Genetics*, 1939, **24**, 709.

Water and Electrolyte Content of Dolphin Kidney and Extraction of Pressor Substance (Renin).

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Former findings on the distribution of electrolytes in the blood¹ and skeletal muscle² of the dolphin (*Tursiops truncatus*), together with the variations revealed in comparisons with terrestrial mammals, actuated a similar examination of the kidneys of the dolphin. The regular occurrence of a pressor substance (renin) in the kidneys of terrestrial mammals stimulated the attempt to demonstrate the presence of such a substance in the kidney of this sea mammal. Therefore, the object of this work was: (1) to determine the water and electrolyte content of the dolphin kidney; (2) to establish the presence of a pressor substance (renin) in this kidney; and (3) to compare the results with corresponding data from the dog as a representative land mammal.

Experimental. One whole kidney weighing 230 g was removed from a live young female dolphin, weighing 80 kg, under sodium phenobarbital anesthesia. The kidney was immediately chilled and frozen. After being wrapped in oiled paper, it was placed in a wide-mouth thermos bottle and shipped by air express from the Marine Studios, St. Augustine, Florida, to our Chicago Laboratories, as described in a previous paper.¹

The dolphin kidney is a compound organ composed of hundreds of small kidney units, each containing a cortex and medulla. Therefore, an aliquot number of units of the whole kidney was taken for chemical analyses, and the remainder for the extraction of the pressor substance. Units amounting to 40 g in weight were analyzed in triplicate for water, fat, chloride, sodium, potassium, calcium and magnesium, using the procedure and methods described by Eichelberger and Bibler.³ These data are given in Table I.

The pressor substance was extracted from 190 g of the kidney units by a method to be described later. To test the pressor activity,

¹ Eichelberger, L., Fetcher, E. S., Jr., Geiling, E. M. K., and Vos, B. J., Jr., *J. Biol. Chem.*, 1940, **133**, 145.

² Eichelberger, L., Geiling, E. M. K., and Vos, B. J., Jr., *J. Biol. Chem.*, 1940, **133**, 661.

³ Eichelberger, Lillian, and Bibler, Walter, *J. Biol. Chem.*, 1940, **132**, 645.

TABLE I.
Water and Electrolyte Content of Dolphin Kidney.
The values are given per kilo of fat-free tissue.

	H ₂ O g	Fat g	Cl mM	Na mM	K mM	Ca mM	Mg mM
	821.0	11.7	65.3	84.2	56.8	1.53	6.3
	Kidneys from 20 normal dogs. ³						
Mean	802.2	19.7	67.7	82.6	58.3	2.16	5.7
σ^*	5.6	9.0	5.3	5.8	4.8	0.53	0.5

*Standard deviation.

the purified extract was injected intravenously into an unanesthetized dog and also into a dog under nembutal anesthesia. Blood pressure of the unanesthetized dog was recorded kymographically by means



CHART 1.

Unanesthetized dog. Wt 11.6 kg. Upper record shows time in intervals of 5 seconds. Middle curve represents femoral blood pressure. (1) Normal blood pressure, 154 mm Hg. (2) 4 cc extract from dolphin kidney (equivalent to 20 g fresh kidney tissue) injected intravenously, blood pressure 228 mm Hg. "X" signifies washings of the needle.

of a gauge 18 needle in the femoral artery connected with a cannula and a mercury manometer. This tracing is shown in Chart I. It will be noted that the control blood pressure of the 11.6 kg dog was 154 mm Hg. After the injection of 4 cc of extract, equivalent to 20 g of fresh kidney tissue, the blood pressure rose to 228 mm Hg. and was still at 212 mm at the end of 10 min.

Blood pressures of the anesthetized dogs were recorded by canulas in the carotid artery connected directly to a mercury manometer. This tracing is shown in Chart 2. It will be noted that the control blood pressure of the dog under nembutal anesthesia was 152 mm Hg. After the intravenous injection of 2 cc of extract, equivalent to 10 g of fresh kidney tissue, the blood pressure rose to 184 mm Hg. The blood pressure of the dog returned quickly to normal evidently because the amount of extract injected was too small. Another injection of 2 cc of the extract caused a second rise to 194 mm Hg. After a third injection of the extract, the blood pressure rose from 166 mm to 200 mm Hg. and persisted at a level of 172 mm for 10 minutes when the experiment was terminated.

These results show that a pressor substance (renin), which functions like the renin from dog or pig kidney, was isolated from the dolphin kidney. (1) The control blood pressure of a 8.9 kg anesthetized dog was 118 mm Hg. After the intravenous injection of

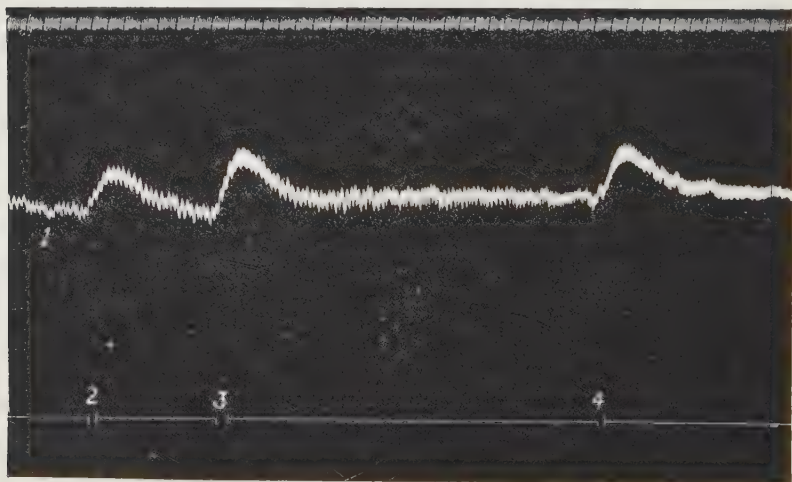


CHART 2.

Anesthetized dog. Wt. 8.2 kg. Records as in Chart 1. (1) Normal blood pressure, 152 mm Hg. (2) 2 cc extract from dolphin kidney (equivalent to 10 g fresh kidney tissue) injected intravenously, blood pressure 184 mm Hg. (3) Second injection of 2 cc of extract, blood pressure 194 mm Hg. (4) Third injection of 2 cc of extract, blood pressure 200 mm Hg.

2 cc of extract made from dog kidney (equivalent to 10 g fresh kidney tissue) the blood pressure rose to 176 mm Hg and was still at 172 mm at the end of 30 min. (2) The control blood pressure of an 8.5 kg anesthetized dog was 110 mm Hg. After the injection of 2 cc extract made from pig kidney (equivalent to 10 g fresh kidney tissue) the blood pressure rose to 240 mm Hg. The blood pressure remained at 170 mm for 15 min., after which another injection of 1 cc extract caused a second rise to 200 mm Hg, which again continued at a level of 170 mm for 15 min. After a third injection of 2 cc extract the blood pressure rose to 190 mm Hg.

Comments. When the content of water and electrolytes in the kidneys of dolphins was collated with comparable data obtained from the kidneys of dogs (Table I), the only difference was the higher water content in the dolphin kidney. The values for sodium and chloride found here, as well as in dog kidneys, are too high to be accounted for by the extra water, assuming that this water is extracellular and contains the concentration of sodium and chloride expected in extracellular fluids. Therefore, it must be assumed that certain cells of the dolphin kidney engaged in the reabsorption of chloride from the glomerular filtrate must contain chloride as well as sodium. Further, the analytical data reflect either of two possibilities: (1) the existence in the lumen of the nephron of an additional fluid phase, varying in composition as it passes down the renal tubules, which must not be different from that found in dogs or else the analytical results would have been decidedly different; (2) if the chloride and sodium concentration representing the extracellular spaces of the kidney is low, as found in the skeletal muscle,² then the sodium and chloride concentration in the contents of the collecting tubules must be considerably higher than that found in the dog.

The potassium figures, as in dog kidneys, indicate that the intracellular phase of the dolphin kidney is relatively small and of the same comparative volume. In view of the complexity of the system and the different kinds of kidneys, it is surprising that the data for dog and dolphin kidneys exhibit the degree of consistency portrayed in Table I.

Conclusions. 1. The values for the water content and electrolyte concentrations of a normal dolphin kidney were as follows: Total water, 821.0 g; chloride, 65.3 mM; sodium, 84.2 mM; potassium, 56.8 mM; calcium, 1.53 mM; and magnesium, 6.3 mM per kg of fat-free tissue.

2. A pressor substance was isolated from the kidney of the dolphin which behaved chemically and physiologically like the renin prepared from the kidney of the dog or pig.

Reduction of Arterial Hypertension by Subcutaneous Implantation of Kidney Tissue.*

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We have obtained evidence that the kidney plays an important rôle not only in the production of the hypertension but also in its elimination.¹ To elucidate further the nature of this relationship, we have investigated the effect of subcutaneous implantation of kidney tissue in normotensive and hypertensive dogs.

Blood pressures of trained unanesthetized dogs were taken using the Hamilton manometer,² according to a technique previously described by us.³ After adequate training, the diastolic pressure of most of our dogs became constant at 75 or 80 mm Hg and these were classed as normotensive. Hypertension was induced in 8 dogs by partial occlusion of the main renal arteries, using the Goldblatt technique.⁴

After a preliminary period of observation during which the arterial pressure was relatively constant, 10 to 15 g of kidney tissue obtained from normotensive dogs was implanted under local anesthesia in the subcutaneous tissue of normotensive and hypertensive dogs. On the third day thereafter the necrotic mass of partially autolyzed kidney was expelled from the wound or was removed. Usually 10 or 20 cc of a sero-purulent fluid escaped from the wound at this time. After removal of the necrotic kidney tissue, the animal improved rapidly.

In the 8 dogs with hypertension of renal origin persisting 47, 91, 100, 140, 182, 200, 400 and 29 days respectively, implantation of kidney tissue resulted in a fall in blood pressure beginning on the second day, reaching a low on the third day and returning to the hypertensive level in the fourth to fiftieth days. The blood pressure returned to the hypertensive levels in 1, 50, 20, 4, 5, 7, 3 and 40 days

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† Now in San Francisco.

¹ Rodbard, S., and Katz, L. N., *Am. J. Med. Sci.*, 1939, **198**, 602.

² Hamilton, W. F., Brewer, J., and Brotman, I., *Am. J. Physiol.*, 1934, **107**, 427.

³ Katz, L. N., Friedman, M., Rodbard, S., and Weinstein, W., *Am. Heart J.*, 1939, **17**, 334.

⁴ Goldblatt, H., Lynch, J., Hanzal, R., and Summerville, W., *J. Exp. Med.*, 1934, **59**, 347.

respectively. The average duration of the fall was 16 days. In the latter 2 dogs, re-implantation of kidney tissue 35 and 330 days after the return of hypertension resulted in a second fall in blood pressure with a return to hypertensive levels persisting for 4 and 36 days respectively after re-implantation. The maximum drop in diastolic pressure amounted to 25 to 50 mm Hg in the 10 experiments and was sustained for the periods indicated. The non-protein nitrogen of the blood remained at normal levels during and subsequent to the depression in arterial pressure.

In 6 normotensive dogs the implantation of kidney tissue had no effect on the blood pressure. The blood pressure remained at the normotensive levels for the duration of the experimental period up to 38 days. Renal ischemia was then produced in 3 of these dogs, 24, 38 and 7 days respectively after the implantation was performed. In all 3 a definite persistent rise in arterial pressure was observed to follow this operation.

In 3 dogs with renal hypertension the production of an infusorial earth abscess had no significant effect on the blood pressure. This abscess was produced by injecting 1 gm of infusorial earth in 10 cc of saline subcutaneously. The large abscess which resulted was opened on the third to fourth day, at which time 25 to 50 cc of pus escaped. The animals improved rapidly following the opening of the abscess.

Our results suggest that during destruction of transplanted kidney tissue, a drop occurs in the blood pressure to normotensive levels in dogs with renal hypertension. No fall in blood pressure occurred following production of an infusorial earth abscess. This is in line with the report⁵ that extensive cellulitis does not lower the pressure of renal hypertensive dogs. Since no fall in blood pressure occurred after implantation into normotensive dogs, the effect seems to be confined to the hypertensive animals. It may persist for long periods beyond the time of removal of the kidney tissue. These facts indicate that the process is not a simple depressor action from absorbed depressor substances but more likely is in the nature of an antagonist to the renal hypertension mechanism. These results are in accord with independent and somewhat different experiments recently reported.^{6,7} A striking difference exists in the two types of ex-

⁵ Wakerlin, G. E., Gaines, W., and Mosny, S. D., *Proc. Am. Physiol. Society*, New Orleans, p. 191, 1940.

⁶ Harrison, T. R., Grollman, A., and Williams, J. R., *Am. J. Physiol.*, 1940, **128**, 716.

⁷ Page, I. H., oral presentation at the American Physiological Society meeting in New Orleans, 1940.

periments in that no blood NPN elevation occurred in our experiments.

Implantation of liver, skeletal muscle, cardiac muscle, spleen, and boiled kidney had only a transient depressor effect upon the blood pressure of hypertensive dogs.

Experiments are under way to determine the nature of this renal principle and to discover whether or not similar effects can be obtained with other tissues.

Summary. Our results suggest that transplanted kidney tissue undergoing degeneration exerts an antagonistic action upon the renal hypertension mechanism.

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Effect of Serum Proteins on the Polarographic Curve.*

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The first attempt to apply the polarographic method to cancer diagnosis was reported by Brdicka.¹ Since then various others have used this technique as a test for the detection of changes in cancer sera.²⁻⁴ A difference has been demonstrated between normal and cancer blood by these workers, but it appears that this difference is chiefly a statistical one. Our own results fully confirm this difficulty. In a survey of 150 cases, individuals were divided into 3 general groups: normal, non-cancerous diseases and cancer.⁵ The values obtained were expressed as the height of the polarographic curve. There was considerable overlapping in all groups, making it impossible to distinguish any individual case as being representative of normal, cancerous or non-cancerous.

During the course of these studies, serum proteins were determined in some of the specimens which also were examined polarographically. There appeared to be a general parallelism between the amount of the serum protein and the height of the polarographic

* Supported by the Jonathan Bowman Cancer Fund and the Wisconsin Alumni Research Foundation.

¹ Brdicka, R., *Nature*, 1937, **139**, 330.

² Bergh, F., Henriques, Q. M., and Wolffbrandt, C. G., *Nature*, 1938, **142**, 212.

³ Wedemeyer, H. E., and Daur, T., *Z. f. Krebs.*, 1939, **49**, 10.

⁴ Walker, A. C., and Reimann, S. P., *Am. J. Ca.*, 1939, **37**, 585.

⁵ Rusch, H. P., Klatt, T., Meloche, V. W., and Dirksen, A. J., in press.

curve. A more extended survey was therefore made of various types of pathological as well as normal bloods, and the polarographic results correlated with variations in the albumin and globulin content as determined by chemical methods. A brief description of the apparatus and the principles underlying its operation has been recently reported by Walker and Reimann.⁴

The blood samples were obtained from fasted subjects. After clotting the serum was withdrawn and aliquots taken for the determination of the serum proteins and for the polarographic test. The serum proteins were determined by Greenberg's colorimetric method. The aliquot used for the polarographic test was treated as follows: 0.1 cc of serum was hydrolyzed by adding 2.5 cc of a 0.05N HCl solution containing 2.5 mg pepsin and incubating at 40°C for 15 min. 0.1 cc of this mixture was then added to 5 cc of a buffer solution which was composed of 10 cc 1N NH_4OH ; 10 cc 1N NH_4Cl ; 10 cc 0.01N cobaltic chloride and 10 cc water. Stock solutions of the various constituents of this buffer were kept in separate bottles and were added together in appropriate amounts just prior to each test. The polarographic measurements were made immediately after the test solutions were prepared.

In order to determine which fraction of the serum protein gave the typical curve, the proteins were precipitated from aliquots of serum with $(\text{NH}_4)_2\text{SO}_4$ and the precipitate divided into albumin and globulin fractions by solution in dilute salt and by coagulation of the globulin on dialysis against distilled water. The crude protein fractions were then replaced separately, and in combination, into appropriate amounts of deproteinized serum and polarographic measurements made as before. Only the albumin fraction yielded a typical curve; the characteristic waves obtained with intact serum were not observed in the globulin fraction, nor did the addition of globulin materially alter the shape of the albumin curve.

TABLE I.
Relation of Level of Serum Albumin to Polarographic Curve.

No.	Diseases	Albumin %		Height of polarographic curve in mm	
		Avg	Range	Avg	Range
7	Nephritis	2.1	1.3-3.1	21.0	13.5-28.0
3	Vomiting of Preg.	2.2	2.0-2.5	22.5	20.5-23.5
3	Osteomyelitis	2.5	1.4-3.6	25.0	13.5-36.0
6	Cancer	2.8	2.5-3.3	26.0	23.0-29.5
22	Miscellaneous*	2.6	1.1-3.8	25.8	17.5-34.5
18	Cardio-vascular	3.0	1.8-4.1	28.0	18.2-34.5
7	Normal	3.5	3.6-4.0	36.5	28.0-44.5

*There were no more than 2 of the same disease in this group.

There was a total of 66 cases in this series, the results of which are listed in Table I. When the average results of the serum from various diseases were compared, a direct correlation was found between the level of serum albumin and the height of the polarographic curve. There was, however, a considerable overlapping of the individual cases in each group (Table I). Nevertheless, the individual results also demonstrated a very close parallelism between the amount of the serum albumin and the polarographic response (Fig. 1). Wedemeyer and Daur have reported a similar correlation.³

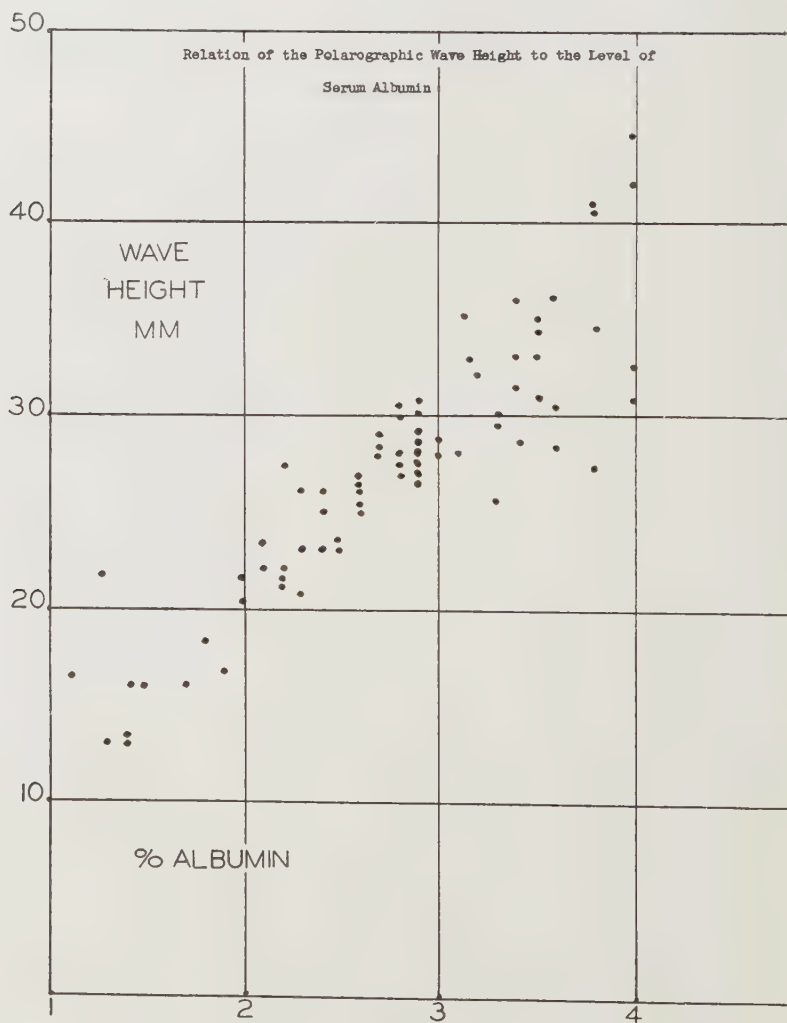


FIG. 1.

The use of the polarograph as a method for cancer diagnosis depends on the measurement of certain sulfur-containing amino acids present in the proteins in the blood serum. The level of these constituents is reported to differ in normal and cancer sera. Brdicka explains the production of the polarographic curve as being due to the catalytic liberation of hydrogen at the dropping mercury cathode induced by the S-H group of cysteine and the S-S linkage of cystine. While this reaction is said to be catalytic, the amount of hydrogen deposited is quantitatively proportional to the concentration of these amino acids. Brdicka also demonstrated that the cystine group gives a wave height twice that for cysteine when equivalent molecular concentrations are used.^{1,6,7} Various workers have determined the cystine content of serum globulin as being about 1.5-3.5% and that of serum albumin from 2.5-6.0%. If we take the usual figures given for the level of albumin as from 4-5 g per 100 cc and those for globulin as 2-2.8 g per 100 cc, we find that the cystine content of the albumin from 100 cc of serum is approximately 0.1-0.3 g while that for the globulin of the same amount of serum is about 0.03-0.09 g. These figures may, in part, explain our findings in regard to the curves obtained with these two separated fractions.

It is interesting to note in passing that various workers have reported a higher sulphydryl content in rapidly growing tissues than in those proliferating slowly.⁸ This has been demonstrated to be the case in embryonic cells, root tips as well as in certain tumors.

Summary. The effect of the serum proteins on the polarographic curve was made on blood sera obtained from 66 normal and pathological individuals. The height of the polarographic curves was found to be directly proportional to the level of the serum albumin. It is obvious, therefore, that the polarographic method is of limited value in cancer diagnosis since changes in serum albumin are by no means specific for neoplastic diseases.

⁶ Brdička, R., *Nature*, 1938, **142**, 617.

⁷ Brdička, R., *Klin. Wochschr.*, 1939, **18**, 305.

⁸ Reimann, S. P., and Hammett, F. S., *Am. J. Ca.*, 1936, **26**, 554.

On the Fate of Ingested Pectin.

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Although pectin is being used for the treatment of certain types of diarrhea, and for other purposes in man, relatively little is known regarding its fate in the alimentary tract. We have been able to find only one report in which the fate of pectin ingested with a mixed diet was studied. Schneider¹ prepared from apple marc a pectin which according to his analysis yielded 35.9% pentosan and 45.8% galactosan. He fed the pectin with a mixed diet very low in cellulose to several human subjects, and found the "coefficient of digestibility" for the pentosans to be 88.7%, and for the galactosans, 76.8%. He also found that intestinal bacteria decompose the hemicelluloses of apple marc. We have studied the fate of pectin fed to dogs with a mixed diet containing no cellulose, and to the same dogs while fasting.

Methods: The pectin used in this study was a very pure citrous pectin obtained from the Research Department, California Fruit Growers' Exchange. By the Link² method 18.2% CO₂ equivalent to 72.8% uronic anhydride was liberated. By the A. O. A. C. method³ for the determination of furfural, 1 g yielded 0.275 g of phloroglucide equivalent to 0.14528 g of furfural. (Uronic acids as well as pentoses yield furfural, hence this factor is not characteristic of all pectins, but must be determined for the particular pectin sample employed.) The methoxyl content was 9.5% and the jelly grade was 180. By the calcium pectate method,⁴ 1 g yielded 110% pectic acid as calcium pectate, on the basis of ash and moisture-free pectin. The foregoing are the average results of a number of analyses made by us. When 5 g of pectin was added to 100 g of feces, 98% could be recovered by the uronic acid method, 94% by the furfural method, and 97.6% by the pectic acid method.

Four dogs weighing from 25 to 35 lb were placed, for the first absorption period, on a mixed diet low in crude fiber (as a control), consisting daily of 200 cc of milk, 200 g of hamburger, and 100 g

¹ Schneider, E. C., *Am. J. Physiol.*, 1912, **30**, 258.

² Dickson, A. D., Otterson, H., and Link, K. P., *J. Am. Chem. Soc.*, 1930, **52**, 775.

³ *Method of Analysis*, Association of Official Agricultural Chemists, p. 344, 4th Ed., 1935.

⁴ Joseph, G. H., personal communication.

TABLE I.
Pectin Recovered from Feces During 7-day Absorption Period as Estimated by 3 Chemical Methods.

Dog No.	Exp. No.	Feces wt (dry) for experimental period, g	Recovery of pectin by furfural method		20 g of pectin per day given with a mixed diet.		Recovery of pectin by uronic acid method		Recovery of pectin by calcium pectate method	
			g	%	g	%	g	%	g	%
I	1				16.24*	11.55				
	2	151			13.30	9.50	14.00	10.00		
	3	113			11.36	8.12	17.40	12.42	5.13	3.66
II	1				12.68	9.06	20.23	14.45		
	2	145			12.05	8.62	15.12	10.80	10.00	7.14
	3	101			11.71	8.37				
III	1				10.00	7.14	4.33	3.09		
	2	79			6.89	6.35	1.51	1.08		
	3	47†							0.00	0.00
IV	1				12.14	8.68	20.80	14.84		
	2	168			12.59	9.00	12.22	8.74	2.19	1.56
	3	95							4.33	3.09
Avg		112.4			11.89	8.64	13.20	9.43		
					20 g of pectin per day given during fasting.		123.80	88.48	120.80	86.30
		197			127.50	91.23	105.30	75.20	95.00	67.80
II	1	188			105.00	75.02	37.40	26.70	32.50	23.06
III	1	48†			34.80	24.82	4.27	3.05	1.80	1.29
IV	1	22†			3.15	2.25	67.69	48.36	62.52	44.61
Avg		144			67.86	48.33				

*Calculation: 140 g of pectin was fed, and is represented by 20.3 g of furfural.
or 16.24 g of pectin.

†Gave only one specimen during experimental period.

However, 2.345 g of furfural returned, or 11.55%.

of liver. During the second period, 20 g of pectin was added daily. For a third period, the dogs were given pectin alone; 20 g dissolved in 500 cc of water was given by stomach-tube. The absorption periods were 7 days in length. Specimens of feces produced by the dogs during each period were collected, dried and pooled, and pectin or its degradation products determined quantitatively as furfural, uronic, and pectic acids.

When the dogs were fed the basal diet alone, no pectic acid was found in the feces, but a total of from 0.37 to 0.42 g of furfural and from 0.72 to 0.90 g of uronic acid was obtained from the different dogs during the 7 day test period. The quantity of furfural and uronic acid obtained during the control period was subtracted from the total obtained during the pectin-feeding period.

Results: Table I shows the quantity of pectin recovered in the feces as determined by the 3 chemical methods. When pectin was added to the mixed diet, furfural estimation indicated a recovery of 8.64%, uronic acid estimation 9.43%, and pectic acid estimation 3.09% (averages of the 4 dogs.) When pectin was given during fasting, furfural estimation gave a return of 48.33%, uronic acid estimation 48.36%, pectic acid estimation 44.61% (averages of the 4 dogs).

Analysis of the data proved to be very interesting. When one adds pectin to a mixed diet practically 90% disappears, and of the amount recovered in the feces only about a third may be obtained as pectic acid. When given during fasting, about 50% disappeared. In this case, dogs I and II defecated frequently, and the recovery of pectin ranged from about 70 to 90%; whereas in dogs III and IV, which defecated only once during 7 days, the amount of pectin recovered ranged from about 2 to 25%. Essentially the same results have been communicated to us by Drs. L. A. Crandall and H. K. Murer. In addition, the results indicate that the decomposition is carried further when pectin is added to a mixed diet than when given alone, for only about a third of the amount which may be recovered in the feces in the former case may be obtained as pectic acid, while in the latter practically all the pectin recovered may be obtained as pectic acid. Decomposition may be also furthered if the pectin fed is retained for longer periods.

Summary. In the dog when 20 g of pectin was fed per day, with a mixed diet over a 7 day period, an average of 90% of the pectin was decomposed; when fed during fasting an average of only 50% was decomposed. These observations, however, may not be applicable to man.

Excretion of Gonadotropic and Estrogenic Hormones in Urine During Normal Menstrual Cycle.

E. V. HAAM AND N. O. ROTHERMICH.

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None of the methods of bioassay for the urinary gonadotropins or estrogens is better than roughly quantitative. As long as definite standards of technic are not available the various absolute values claimed in the literature cannot be compared and no definite statements as to right or wrong can be issued. However, normal and abnormal fluctuations of these hormones during the menstrual cycle should be recognized with some degree of uniformity regardless of the absolute values obtained by the various methods. Our former belief in a single excretion peak of urinary gonadotropins during the menstrual cycle seems disproven by D'Amour, Funk and Liverman,¹ who showed as many as 3 excretion peaks in daily assays of urinary gonadotropins from normal women. Our previous conception of the premenstrual height of estrin excretion has been refuted by the careful investigation of Gustavson and co-workers,² who showed that as early as 5 days after the onset of menstruation a peak in estrin excretion curve can be found. This demonstrated that more data on the normal menstrual cycle of women are needed in order to establish the correlationship between the excretion of gonadotropic and estrogenic hormones. We have examined the urine of 3 healthy women during a complete menstrual cycle for gonadotropic and estrogenic substances and wish to report briefly on the results:

Method. Twenty-four-hour specimens of urine were collected from 3 women between the ages of 20 to 30 who by careful history did not show any evidence of menstrual disorders. In all 3 women menarche set in between 11 and 13 years, and menstruation occurred every 28 days, lasting from 4 to 6 days. There was no excessive flow, no menstrual pain or premenstrual tension. The specimens were kept on ice during collection and were assayed every 48 hours. Urinary gonadotropins were determined by the method of Levin and

¹ D'Amour, F. E., Funk, D., and Liverman, H., *Am. J. Obst. and Gynec.*, 1939, **37**, 940.

² Gustavson, R. G., Mason, L. W., Hays, E. E., Wood, T. R., and D'Amour, F. E., *Am. J. Obst. and Gynec.*, 1938, **35**, 115.

Tyndale,³ using the uterine weight of immature mice as indicators. After precipitation with tannic acid had been completed, the remain-

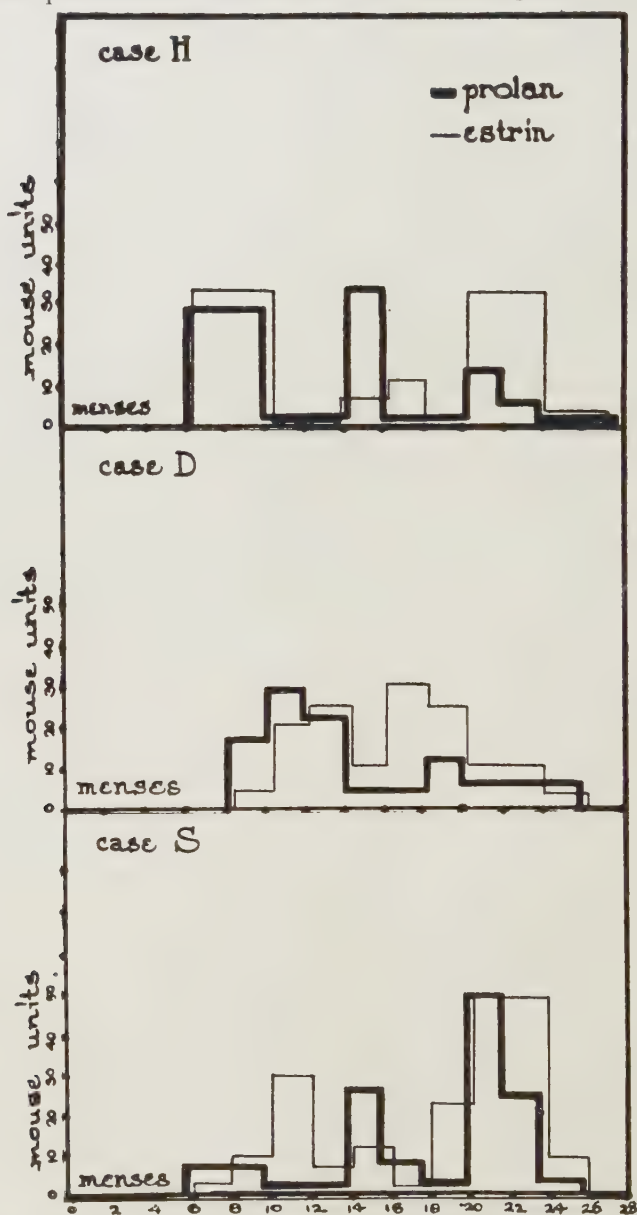


FIG. 1.

Curves of daily excretion of urinary gonadotropins and estrogens during the normal menstrual cycle.

³ Levin, L., and Tyndale, H. H., *Endocrinology*, 1937, **21**, 619.

ing urine was acidified, hydrolized, and extracted with benzene, following the method of Smith and Smith.⁴ The residue was dissolved in glyocol propionate and injected into immature white mice following the suggestion of Lauson, Heller, Golden and Sevringhaus.⁵ We have repeatedly convinced ourselves that the tannic acid precipitates were free of any estrogenic substance.

Results. The curves depicting the cyclic excretion of estrogen and gonadotropin are pictured in Fig. 1. It appears that 1 to 3 peaks of prolactin excretion may occur in a single cycle. The excretion of estrin either parallels or in some instances precedes the prolactin excretion. Two distinct peaks have been observed in all our cases. In Patient D they were so close to each other that a separation may seem arbitrary. If we wish to deduce from the appearance of the hormone levels in the urine the time and incidence of ovulation, we must agree with Gustavson's statement that "the time of ovulation may vary considerably in different individuals, and that the corpus luteum may require varying periods of time to reach its full development and activity."

11458 P

Non-Identity of Gray Hair Produced by Mineral Deficiency and Vitamin Deficiency.

ALFRED H. FREE. (Introduced by Victor C. Myers.)

From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland.

The recent recognition of a specific organic food factor^{1,2} necessary for the maintenance of the black fur of black or piebald rats has raised the question as to whether the phenomenon of graying noted by older workers^{3,4,5,6} particularly in rats on an exclusive milk diet is due to this cause or is due to a specific mineral deficiency. Jukes and

⁴ Smith, G. V., and Smith, O. W., *Am. J. Physiol.*, 1935, **112**, 340.

⁵ Lauson, H. D., Heller, C. G., Golden, J. B., and Sevringhaus, E. L., *Endocrinology*, 1939, **24**, 35.

¹ Morgan, A. F., Cook, B. B., and Davison, H. G., *J. Nutrition*, 1938, **15**, 27.

² Lunde, G., and Kringstad, *Z. physiol. Chem.*, 1938, **257**, 201.

³ Hartwell, G. A., *Biochem. J.*, 1923, **17**, 547.

⁴ Kiel, H. L., and Nelson, V. E., *J. Biol. Chem.*, 1931, **93**, 49.

⁵ György, P., *Biochem. J.*, 1935, **29**, 741.

⁶ Gorter, F. J., *Z. Vitaminforsch.*, 1935, **4**, 277.

Richardson⁷ have pointed out that milk is not a good source of filtrate fraction vitamins.

The black fur of young piebald rats fed an exclusive diet of either powdered whole milk or of fresh certified milk rapidly and uniformly became gray. Addition of a supplement of 0.25 mg of iron, 0.05 mg of copper and 0.05 mg of manganese cured the grayness if it had developed or prevented any change in the color of the fur of rats which were fed milk with the above mineral supplements from the time of weaning. Attempts to ascertain which of the 3 metals is responsible have not at present yielded entirely conclusive results.

Rats developing a nutritional anemia show a marked diminution of appetite and the possibility exists that the graying of rats may be due to a diminished intake of vitamin accompanying the reduced intake of the vitamin-poor milk. This has been disproven since in 2 groups of rats fed isocaloric amounts of milk, those supplemented with iron, copper and manganese did not develop grayness whereas those fed milk alone became quite gray. It has also been found that rats that have developed grayness will become black when supplemented with the above minerals whereas the isocaloric controls without minerals remain gray. Rats, following the production of gray hair by a milk diet, when supplemented with a rice bran extract* rich in the factors of the B complex very slowly regained their black color. However, control rats fed equal quantities of the ashed rice bran extract likewise slowly became black. Hemoglobin regeneration slowly occurred in both the rats receiving rice bran and in the rats receiving rice bran ash. This indicates that the effect with milk-fed gray rats is entirely due to inorganic substances in the preparation.

Rats fed a synthetic diet consisting of purified casein, cane sugar, butter, salt mixture, cod liver oil, and supplements of synthetic vitamins B₁, B₆, and riboflavin† developed gray hair. The hemoglobin level in these rats was normal. Iron, copper, and manganese supplements in no way altered the response of these animals. However, rice bran extract in the same amount used above was effective in the cure of these animals. With the vitamin-deficient animals the ash of the rice bran was without effect. Rats given a daily ration of 50 cc of milk plus 1 g of the synthetic diet showed no graying or were cured when placed on such a diet after production of the gray hair either by mineral or vitamin deficiency.

All of the present evidence based on experiments with approxi-

⁷ Jukes, T. H., and Richardson, G. A., *J. Agr. Res.*, 1938, **57**, 603.

* Obtained through the courtesy of Dr. Paul György.

† Kindly supplied by Merck and Co., Inc., Rahway, New Jersey.

mately 50 rats seems to indicate that graying of black hair in rats may result from a deficiency of a factor or factors present in the vitamin B complex and may also result from a deficiency of iron, copper, and manganese.

11459

Effects of Testosterone Propionate on Female Roller Canaries under Complete Song Isolation.

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MILTON METFESSEL.

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It is commonly assumed that secondary sexual characteristics are conditioned by the secretions of the respective gonads of each sex. Singing in canaries is normally limited to the male of the species and so may be considered as a male secondary sexual characteristic.

Baldwin and Goldin¹ indicated that when testosterone propionate was administered to the female viviparous teleost, *Xiphophorus helleri* Heckel, the male secondary sexual characteristics were induced in all cases. Noble and Wurm² treated adult females and immatures of both sexes of the black-crowned night heron, and produced male sexual behavior. They concluded that the differences between the sexual behavior of adults of this form seem to be regulated only by proportionate differences in the amounts of male hormone normally found in these birds. Allee and Collias³ reported crowing in hens treated with testosterone propionate, and cessation of this crowing soon after the treatment was stopped, indicating the dependence of this behavior on the male hormone.

Leonard⁴ treated female roller canaries with testosterone propionate and reported that they produced song that differed from normal male song only in the greater sound volume produced by the males. He also stated that his best results were obtained when the females were "isolated" by putting them in individual cages but keeping them in

¹ Baldwin, F. M., and Goldin, H. S., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **42**, 813.

² Noble, G. K., and Wurm, M., *Anat. Rec.*, 1938, **72**, Sup. 1, 60.

³ Allee, W. C., and Collias, N., *Anat. Rec.*, 1938, **72**, Sup. 1, 60.

⁴ Leonard, S. L., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 229.

the same room.* Shoemaker⁵ likewise induced song in treated female canaries, and observed that the failure of these birds to tread receptive females may reflect the lack in testosterone propionate of the capacity to imitate the complete chain of events caused by the normal testicular hormone, and not to a lack in the nervous system, as two untreated females were observed to copulate like males.

From a behavior standpoint, Lashley⁶ indicated that in the rat, hormone action seems to activate some central nervous mechanism to maintain excitability or activity. Moore⁷ made observations on castrated and transplanted rats that showed the transforming power of the gonads of one sex over the psychic nature of the opposite sex. He concluded that this psychic behavior, absolutely distinct in itself, lends great weight to the idea of transformed sexual nature.

Experimental. The canaries used were raised out of doors by a local breeder, and were all past one year of age. Each bird was put in its own soundproofed, ventilated cage⁸ to preclude any effects of song environment in addition to the administration of the testosterone propionate. These birds were isolated for observation over a preliminary period of 2 weeks before administration to assure the absence of any song. A crystal microphone was placed in each cage and provision made for recording any sound of the birds on aluminum discs. An operator listened to the sounds at a control board in another room. As soon as any sounds were heard, samples were recorded. Lights were on in the cages at regular daily intervals corresponding in length to the normal waking hours of the birds.

Six birds were used in this problem, 4 for treatment with testosterone propionate, and 2 to act as oil-treated controls. Injections were made daily at the same hour, each bird receiving 2.5 mg of testosterone propionate† per injection. Thirteen subcutaneous injections in alternate breast regions constituted the extent of the experimentation. The controls were given similar treatment with sesame oil.

Results. While the microphones, amplifiers and recording disc were in readiness for recordings of the voices and calls of the treated birds from the start of the injections, the first song calls in any of the treated birds were forthcoming on the twelfth day. The

* From personal communications with the author, S. L. Leonard.

⁵ Shoemaker, H. H., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 299.

⁶ Lashley, K. S., *Psycholog. Rev.*, 1938, **45**, 445.

⁷ Moore, C. R., *J. Exp. Zool.*, 1919, **28**, 137.

⁸ Metfessel, M., *J. Psychology*, 1940, **10**, 177.

† The male hormone, Oreton, was furnished through the kindness of Dr. Max Gilbert of the Schering Corporation.

second bird sang on the thirteenth day, the third bird on the sixteenth day, and the fourth bird on the twentieth day. From these data, it appears that the average length of time in administration of the hormone approximates fifteen days to produce song. Three of the treated birds gradually developed male-like tours¶ that were somewhat of the same pattern and quality, with limited variations. The fourth bird showed a song of a varied pattern that followed the same sequences when the songs were repeated. The songs developed exhibit a small repertoire with poor male quality so far, but with voice that was definitely male in character. Cessation of treatment resulted in a return to the ordinary female calls.

Conclusion. The administration of testosterone propionate to normal adult female roller canaries under conditions of complete song isolation brings forth male-like song in approximately 15 days after first administration, and thus substantiates previous theories.³⁻⁷

11460 P

Isolation of a Murine Neurotropic Virus by Passage of Monkey Poliomyelitis Virus to Cotton Rats and White Mice.*

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Armstrong^{1,2,3} reported apparent transmission of poliomyelitis (Lansing strain) from the monkey to the Eastern cotton rat and to white mice. This report deals with attempts to adapt other strains of poliomyelitis virus to these rodents.

Cotton rats (*Sigmodon hispidus littoralis*) were infected intracerebrally with 5 recognized strains of monkey poliomyelitis virus (RMV, Aycock, Philadelphia, ST Los Angeles, SK New Haven). None of the animals injected with the first 4 strains showed any abnormal symptoms. However, of 2 cotton rats injected with the SK‡

¶ A tour is somewhat analogous to a syllable of language; there are thirteen recognized tours in roller canary song.

* Supported by a grant from the Philip Hanson Hiss, Jr., Memorial Fund.

† Fellow in Dermatology.

1 Armstrong, C., *Public Health Reports*, 1939, **54**, 1719.

2 Lillie, R. D., and Armstrong, C., *Public Health Reports*, 1940, **55**, 115.

3 Armstrong, C., *Public Health Reports*, 1939, **54**, 2302.

‡ Received in its 11th monkey passage through the courtesy of Dr. John R. Paul.

strain 1 died the following day, evidently of trauma; the other one succumbed one week later without observed symptoms. No lesions were present except a markedly congested brain, sterile upon aerobic and anaerobic cultivation. Intracerebral transfer of this brain to another cotton rat resulted in mild nervous symptoms within 2 days, and death the next day. Further passage of the brain of the second cotton rat produced in a third cotton rat flaccid paralysis of both hind legs on the 6th day, followed by death 24 hr later. From the last 2 cotton rats intracerebral transfers of brain suspensions were made to groups of white mice. All injected mice developed complete flaccid paralysis of the hind legs, within 3 or 4 days, followed by generalized paralysis and death.

Subsequent attempts to reproduce passage from monkey to cotton rats and white mice with the original material were unsuccessful. Mouse virus, however, since its isolation, is transmissible from mouse to mouse in an unbroken series. At the time of this writing, *i. e.*, April 24th, 1940, the virus is in its 23rd passage. Over 2500 mice have been inoculated; excepting those injected with virus known to be inactivated or impotent all mice have developed the same characteristic symptoms, with only an occasional recovery, to wit: flaccid paralysis (unilateral or bilateral) of hind legs, seldom of forelegs, occasional encephalitic syndrome, death. The described symptomatology is somewhat similar to that of Theiler's⁴ spontaneous mouse encephalitis, but the two viruses differ in important aspects (degree of virulence, incubation period, routes of infection, age factor, latent immunization, serological reactions). Moreover, mice from a Theiler-immune colony are not protected against infection with the mouse virus.

Stained brain suspensions from paralyzed mice, when examined microscopically, show no characteristic morphological unit. Seeding of blood agar, broth or 10% serum broth results in no visible growth after prolonged aerobic or anaerobic incubation. However, the infectious agent passes through V, N and W Berkefeld filters without appreciable diminution in virulence. It is completely destroyed by heating for $\frac{1}{2}$ hr at 60°C and by exposure to ultraviolet light for 1 min, but resists phenol up to 1% concentration. In glycerin it has remained viable up to 1 month in the icebox.

White mice may be successfully infected by any one of the following routes: intracerebral, intranasal, intraperitoneal, intravenous, subcutaneous and by feeding. Upon intracerebral injection a constant potency of 1:1,000,000 is obtained and an occasional endpoint

⁴ Theiler, M., *Science*, 1934, **80**, 122.

of 1:20,000,000. The maximum incubation period has not exceeded 1 week and may be as short as 48 hr with lower dilutions. Intraperitoneal injection of a dose of 1:1000 of a virus brain suspension uniformly produces paralysis within from 3 to 4 days. As early as 2 hr after introducing the virus into the peritoneal cavity it may be recovered from the brain and blood in both of which it persists until the terminal stage. Virus concentrations were highest in the brain and cord, with the adrenal next. Other organs (spleen and liver) carried smaller amounts of the infectious agent.

The virus fails to induce any symptoms in albino rats, guinea pigs and rabbits following repeated injections of large doses by a diversity of routes. It is easily transferable back to cotton rats, producing regularly paralysis and death in that species. Its pathogenicity for monkeys is questionable. Of 10 monkeys injected intracerebrally with either mouse or cotton rat virus 8 passed through a sharp fever cycle; only 5 animals developed weakness of the extremities and 2 others slight transitory facial paresis. In no case did the symptoms progress to typical spinal paralysis.

In the brain of paralyzed mice diffuse proliferation of glia cells and occasional foci of perivascular cuffing are observed. Severe damage occurs in the cord in both anterior horns, extending from loss of Nissl substance and irregular nuclear staining to a complete breakdown of the nerve cell with subsequent neuronophagia. Microglial proliferation is widespread at some levels as is perivascular infiltration.

Doses of virus ranging from 1:1,000,000 to 1:100 were tested for *in vitro* inactivation by the following sera: monovalent immune sera from monkeys convalescent from infection with RMV, Aycock, or SK virus, hyperimmune horse serum (anti-RMV), pooled human convalescent serum, normal serum from man, monkey and horse and antiviral immune sera against other neurotropic viruses (Theiler-mouse encephalitis, equine encephalomyelitis, rabies, St. Louis encephalitis, herpes). Normal animal and human sera, as well as the other antiviral immune sera, failed to bring about inactivation of the virus as its highest effective dilution (1:200,000); convalescent Aycock and SK monkey sera and convalescent human serum neutralized at slightly lower levels (1:100,000 to 1:50,000). Neutralization extending through a virus concentration of 1:1000 was obtained with the hyperimmune horse serum. RMV monkey convalescent serum failed to neutralize. The above data are based on results obtained in repeated tests. These immunological reactions are consistent with those of SK virus in monkeys, which is

neutralizable by SK and Aycock antiserum but not by RMV convalescent monkey serum.⁵ A discrepancy exists regarding normal human serum which neutralizes SK virus in monkeys but not mouse virus in mice (3 sera tested).

Eight monkeys were immunized with a series of subcutaneous injections of live mouse virus and then tested for cross-immunity by intracerebral injection with 3 different strains of virulent monkey poliomyelitis virus. The results of this experiment were as follows: Three immunized monkeys, subsequently infected with the homologous virus, SK, remained free from paralysis of the extremities; none of the other 5 immunized monkeys, subsequently infected with the heterologous strains (Aycock and RMV), escaped the disease. An equal number of controls, infected with the same virus strains, developed typical poliomyelitis. Neutralization tests with monkey sera obtained at the end of immunization showed various titers of mouse virus neutralizing antibodies; in 6 instances neutralization was obtained in monkey tests against SK, Aycock, and RMV virus (3 with SK, 2 with Aycock, 1 with RMV).

In its 6th mouse passage the virus was cultivated in serum-ultrafiltrate tissue cultures (Sanders⁶) containing embryonic mouse or guinea pig brain or whole minced chick embryo by transferring every 3 days supernatant fluid or whole culture emulsion. The 6th serial passage of embryonic mouse brain cultures produced typical symptoms and death in mice following injection up to 1:10,000,000 dilution of the supernatant culture fluid. Similar passages of guinea pig brain cultures titrated up to 1:10,000. Only traces of virus were recovered from the chick embryo cultures.

⁵ Trask, J. D., Paul, J. R., and Vignec, A. J., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 241.

⁶ Sanders, M., *J. Exp. Med.*, 1940, **71**, 113.

11461

Experimental Arthritis in the Albino Rat Produced by a Group A Hemolytic Streptococcus.*

SIDNEY ROTHBARD. (Introduced by E. L. Opie.)

From the Department of Pathology, Cornell University Medical College, New York City.

Collier¹ described a spontaneous polyarthritis in rats from which no microorganism was cultivated. However, bacteriologically sterile organs produced the disease on reinoculation into other rats. Rhodes and van Rooyen² reported a similar disease in rats from which no bacteria were recovered by aerobic or anaerobic methods. Findlay, Mackenzie, MacCallum and Klieneberger³ also described a spontaneous, infectious arthritis in rats, but they were able to isolate a pleuropneumonia-like organism (L 7) in pure culture which reproduced the arthritis when injected into other rats. Recently Watson⁴ has shown that an acute purulent arthritis can be produced in mice after injections of several strains of hemolytic streptococci.

Because of the interest aroused in this subject by these reports and because a review of the literature reveals no account of the disease in rats due to the streptococcus, it seems advisable to report the production of an acute polyarthritis in the albino rat by the intravenous injection of a hemolytic streptococcus recently isolated from the blood stream of a patient with septicemia.

The streptococcus was of the "matt" variety, Group A and of an unclassified type.† 0.5 cc of an 18-hour broth culture when injected intravenously produced arthritis in 100% of rats weighing from 70 to 100 g while a smaller dose (0.1 cc) produced it in approximately 70% of the animals.

The arthritis appears as early as 48 hours after inoculation. It is multiple in character and new joints develop in succession for 8 days after inoculation. In a few instances the swelling in some joints has decreased during the period of observation while other joints are

* Supported by a grant from the John and Mary R. Markle and the Ophthalmological Foundations.

¹ Collier, W. A., *Geneesk. Tijdschr. Ned.-Ind.*, 1938, **78**, 2845.

² Rhodes, A. J., and van Rooyen, C. E., *J. Path. and Bact.*, 1939, **49**, 577.

³ Findlay, G. M., Mackenzie, R. D., MacCallum, F. O., and Klieneberger, E., *Lancet*, 1939, **2**, 7.

⁴ Watson, R. F., personal communication, 1940.

† Grouped by R. C. Lancefield of the Hospital of the Rockefeller Institute for Medical Research.

becoming involved. The diseased joints are swollen, dusky red in color, hot and painful to palpation. (Fig. 1.) In some animals as many as 8 different joints develop arthritis. The ankle joint is involved most often, the wrist next in frequency and then the tarsal and carpal interphalangeals. Many of the joints healed completely, but others have progressed, and the arthritis has been present for 8 weeks after inoculation. The rats move about with difficulty, drag their hind limbs and appear ill, but as a rule, do not succumb to the infection.

Gross and microscopic examinations have been made up to 7 days after onset of the disease. At this period the joint is enlarged, the periarticular tissues are oedematous and have a mucinous consistency. The synovial membrane is grey yellow in color and covered with a gelatinous exudate. The synovial fluid is in slight excess, viscid and opaque, but not purulent. The cellular content of the

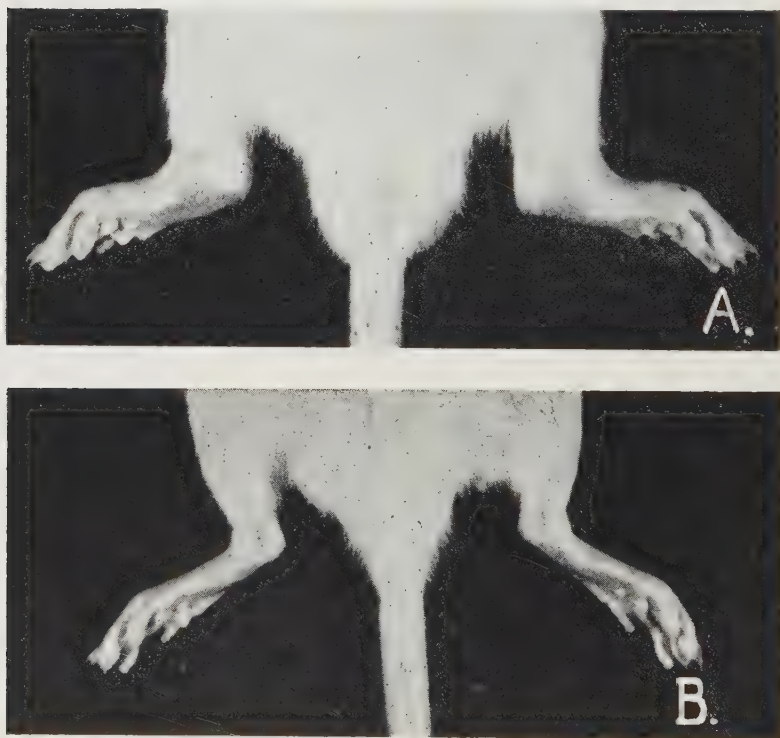


FIG. 1.

Comparison between the ankle joints of a rat with arthritis 7 days after the intravenous injection of 0.5 cc of hemolytic streptococci (A) and those of a normal rat (B).

exudate shows on an average, 65% polymorphonuclear leukocytes and 35% round cells. Streptococci are demonstrable in smears of the synovial fluid and are cultured without difficulty. These organisms, when reinjected into other rats, reproduce the disease. The cartilage and bone show no changes at this stage. Heart blood cultures are positive for 5 days after the intravenous injection. In 5 instances an acute purulent endophthalmitis involving one eye was found.

Microscopically, the periarticular tissues are oedematous with separation of the muscle fibres and fascia. Fibrin and pink-staining fluid are present in the interstitial spaces. The tissues are infiltrated with polymorphonuclear leukocytes which are found in focal collections in some areas, a few monocytes and an occasional lymphocyte. The subsynovial fat shows the same type of cellular reaction that is present in the periarticular tissue. The most conspicuous inflammatory reaction appears in the synovial villi. In some areas the synovial membrane is absent, but in others there is evidence of proliferation of synovial cells. The joint cavities contain considerable cellular debris, fibrin, polymorphonuclear leukocytes and round cells.

A more detailed study of this experimental disease is now in progress.

Summary. An acute multiple arthritis has been produced in 45 of 51 albino rats by the intravenous injection of a Group A hemolytic streptococcus. In the eyes of 5 rats, an acute monocular purulent endophthalmitis was present.

The author wishes to thank Dr. D. M. Angevine for his assistance in this work.

Availability of Glucose for Human Brain Oxidations.*

JOSEPH WORTIS AND WALTER GOLDFARB.

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The ready availability of glucose for brain tissue oxidations has been demonstrated both in the Warburg apparatus (Warburg, Posener and Negelein¹) and in the intact organism (Himwich and Nahum,² Lennox³) Damashek, Myerson and Stephenson⁴ have shown that when the human brain is deprived of this foodstuff during insulin hypoglycemia the oxygen uptake of the brain is correspondingly diminished, and Himwich, Bowman, Wortis and Fazekas⁵ have shown that in the deep coma associated with therapeutic insulin shock brain metabolism approaches zero. Under these conditions in humans the brain potentials have been found to be diminished (Hoagland, Rubin and Cameron⁶) and typical neurologic signs appear. Clinical coma, neurologic signs and the characteristic brain potential changes all disappear after glucose administration. The increased oxygen uptake by the brain after glucose administration had already been demonstrated in dogs (Himwich, *et al.*,⁷), and in humans the neurological signs are alleviated by the administration of as little as 4 g of glucose (Himwich, Frostig, *et al.*,⁸)

In the course of our investigations of the availability of various food substrates for brain metabolism (Wortis and Goldfarb⁹) we have undertaken to establish the metabolic response of the brain to small doses of glucose administered intravenously to schizophrenic

* Aided by a grant from the Havelock Ellis Fund for Psychiatric Research.

¹ Warburg, O., Posener, K., and Negelein, E., *Biochem. Z.*, 1924, **152**, 309.

² Himwich, H. E., and Nahum, L. H., *Am. J. Physiol.*, 1930, **90**, 389.

³ Lennox, W. G., *Arch. Neurol. Psych.*, 1931, **26**, 719.

⁴ Damashek, W., Myerson, A., and Stephenson, C., *Arch. Neurol. and Psychiat.*, 1935, **33**, 1.

⁵ Himwich, H. E., Bowman, K. M., Wortis, J., and Fazekas, J. F., *J. Nervous and Mental Disease*, 1939, 273.

⁶ Hoagland, H., Rubin, M. A., and Cameron, D. E., *Am. J. Physiol.*, 1937, **120**, 559.

⁷ Himwich, H. E., and Fazekas, J. F., *Endocrinol.*, 1937, **21**, 800.

⁸ Himwich, H. E., Frostig, J. P., Fazekas, J. F., and Hadidian, Z., *Am. J. Psychiat.*, 1939, **96**, 371.

⁹ Wortis, J., and Goldfarb, W., *Science*, 1940, **91**, 270.

subjects during therapeutic insulin shock, to serve as a basis of comparison for the availability of other substrates for brain oxidations.

Experimental. The cerebral metabolism was estimated from the arterio-venous differences of O_2 , CO_2 , and glucose. The arterial blood was obtained from either the brachial or femoral arteries, and the venous blood was sampled from the internal jugular vein using the technic described by Myerson, *et al.*¹⁰ The blood samples were analyzed for O_2 , CO_2 (Van Slyke and Neill¹¹), and glucose (Folin and Wu¹²). The velocity of circulation in the peripheral vascular system was estimated with the method of Robb and Weiss.¹³ The metabolism of the brain was estimated after the patients were in hypoglycemic coma, and again at various intervals after the administration of 4 g of glucose intravenously. The latter dose was chosen after some preliminary trial as the minimum amount required to rouse the patients. At the end of the experiment the patients were given the usual large amount of glucose to terminate the treatment.

The summary of data is presented in Table I. In 15 patients the administration of 4 g of glucose intravenously was sufficient to promptly rouse the patients from coma. In 13 experiments the

TABLE I.
Effect of 4 Grams of Glucose Intravenously on Cerebral Metabolism of Schizophrenic Patients in Coma.

Exp. No.	Min. after glucose	Volume % oxygen			Glucose		Circ. Time
		Art.	Ven.	Diff.	Art.	Ven.	
1	10	19.4	15.8	3.6			11
2	5	19.8	13.2	6.6			
3	5	17.9	15.1	2.8	57	57	10
4	9	21.4	13.1	8.3			14
5	5	18.1	8.4	9.6	48	40	12
6	4	19.0	12.3	6.7			
7	4	21.8	14.1	7.7	56	43	15
8	5	20.5	15.9	4.6	69	68	10
9	5	19.2	14.4	4.8	47	43	10
10	6	22.4	15.9	6.5	53	47	10
11	5	20.9	16.1	4.8			
12	5	19.6	14.4	5.2			
13	4	17.1	13.1	4.0	56	62	16
14	4	19.0	16.0	3.0	23	30	
15	4	20.9	17.2	3.7	42	49	
Avg		19.8	14.3	5.46	50	49	12.0
Avg of 60 cases in insulin coma		19.6	16.7	2.9	23	21	11.8

¹⁰ Myerson, A., Halloran, R. D., Hirsch, H. L., *Arch. Neurol. and Psychiat.*, 1927, **17**, 807.

¹¹ Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **51**, 523.

¹² Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, **41**, 367.

¹³ Robb, G. P., and Weiss, S., *Am. Heart J.*, 1932-3, **8**, 650.

oxygen uptake of the brain was increased significantly above the average of a large control series, the average changing from 2.9 volumes % to 5.46 volumes % after glucose administrations. During coma the arterial glucose averaged 23 mg % and the venous sugar 21 mg %. After glucose administration the values were 50 and 49 mg % respectively. The circulation time revealed no change.

The estimation of brain metabolism in these experiments was based on the differences between the concentrations of various substances in the arterial and venous blood. We are in agreement with other investigators that this method does not measure the total metabolism of the brain, and that the observations are affected by changes in blood flow; Abramson, *et al.*,¹⁴ have observed an increased blood flow in the extremities of patients receiving insulin therapy for schizophrenia, and the authors concluded that the evidence strongly suggested that there was an increased blood flow through the brain. They believe that the reduced oxygen uptake found in insulin hypoglycemic coma may be attributable to this possibly increased blood flow through the brain. Abramson's observations, however, in contrast to ours, were made less than 2 hours after insulin injection, preceding the onset of coma. Moreover, it is well known that blood flow through the brain may vary independently of the blood flow in the periphery, and is principally controlled by the arterial blood pressure. The latter gradually falls if the hypoglycemia is not associated with convulsive seizures. Direct observation of blood flow during insulin hypoglycemia in rabbits reveals no significant changes unless convulsions occur (Leibel and Hall¹⁵). In humans a gradual decrease in brain blood flow occurs during insulin hypoglycemia (Loman and Myerson¹⁶). Such a decrease in blood flow indicates that there is probably an even greater diminution of brain metabolism than the diminished arterio-venous oxygen differences recorded by Himwich, Bowman, Wortis and Fazekas¹⁷ would indicate. It will be noted that the arterio-venous difference for glucose did not return to normal values with the return to a normal oxygen difference. This apparent discrepancy can probably be explained on the assumption that the glucose administered is rapidly absorbed by the tissues, including the brain tissue, and that its subsequent utilization within the cell is not accompanied by any further removal of glucose from the

¹⁴ Abramson, D. I., Schesloven, N., Margolis, M. N., and Mirsky, I. A., *Am. J. Physiol.*, 1939, **128**, 124.

¹⁵ Leibel, B. S., and Hall, G. E., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 894.

¹⁶ Loman, J., and Myerson, A., *Am. J. Psychiat.*, 1936, **92**, 791.

¹⁷ Wortis, J., Bowman, K. M., Goldfarb, W., Fazekas, J. F., and Himwich, H. E., *Am. J. Physiol.*, 1940, in press.

blood. Since there is no comparable intracellular storage of oxygen the increased oxygen uptake continues. This supposition is supported by reference to blood sugar curves after the administration of 4 g of glucose intravenously to patients in insulin coma. These show a rapid removal of glucose from the blood, preceding clinical arousal (Fig. 1) with a persistence of clinical recovery long after the blood glucose ceases to show any further considerable drop.

The rapid clinical arousal and the rapid increase in oxygen uptake, however, again confirm the ready availability of glucose as a metabolic substrate for the human brain, and affords a convenient yardstick for comparison with the availability of other foodstuffs for brain metabolism during hypoglycemic insulin coma.

Summary and Conclusions. The availability of glucose for brain oxidations in hypoglycemic insulin coma was studied in human patients. The intravenous administration of 4 g (in 50% solution) invariably aroused the patients and approximately doubled the oxygen uptake of the brain.

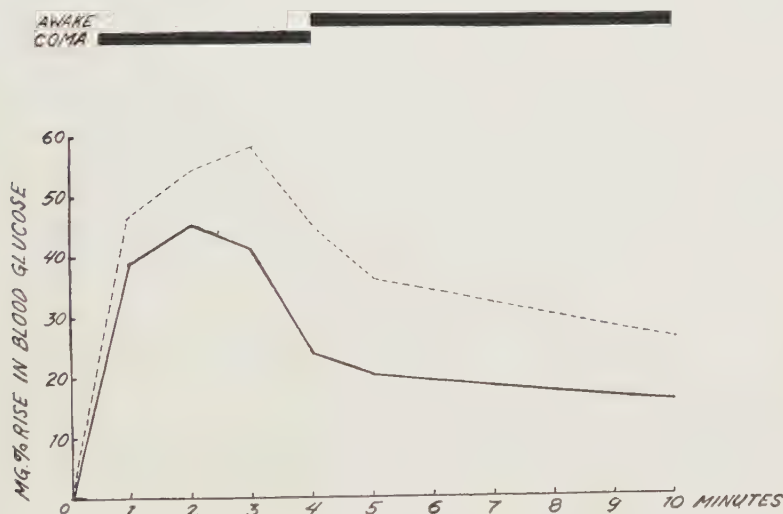


FIG. 1.

The solid line shows the amount of increase of blood glucose (antebrachial vein) following intravenous administration of 4 g of glucose during insulin coma. The dotted line shows the amount of rise above fasting levels in the same patients on non-treatment days. The comatose patients almost invariably roused in about 4 minutes. The curves are based on averages of 13 experiments in coma, and 7 experiments outside of coma.

Presence of a Hitherto Unrecognized Nicotinic Acid Derivative in Human Urine.*

VICTOR A. NAJJAR AND ROBERT W. WOOD. (Introduced by L. Emmett Holt, Jr.)

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In the course of some studies on the excretion of thiamin in urine by means of the thiochrome method, using the procedure of Hennessy and Cerecedo,¹ it was noted that treatment of the KCl eluate of urine with alkali, even in the absence of ferricyanide, yielded a small amount of a substance soluble in butyl alcohol which gave a bluish fluorescence with ultraviolet light. This fluorescence could be distinguished from that given by thiochrome even with the naked eye, being a whitish blue without any tinge of violet. Specimens of urine from a large series of normal individuals of various ages were found to exhibit such fluorescence in slight degree. A patient receiving nicotinic acid therapy, however, was found to excrete it in large amount, suggesting that nicotinic acid was the precursor of this substance. Following this observation the effect of taking nicotinic acid was studied in normal individuals, and it was found that a dose of 50 mg of nicotinic acid, given to an adult, produced a prompt increase in the excretion of the unknown material. An increase in its concentration in the urine could be detected within an hour, and persisted for 4 to 6 hours.

We have attempted to identify the unknown urinary constituent by studies of the fluorescence of 27 different pyridine derivatives.† These compounds were dissolved in 25% KCl solution, both with and without treatment with alkali; the aqueous solution was then extracted with butyl alcohol, the alcoholic extract being tested for fluorescence with ultraviolet light. The following substances were tested:

nicotinic acid
nicotinic acid amide

* This study was aided by a grant from Mead Johnson and Co., Evansville, Ind.
1 Hennessy and Cerecedo, *J. Am. Chem. Soc.*, 1939, **61**, 179.

† Most of these compounds were furnished us through the courtesy of Dr. Charles E. Bills. Dr. William A. Perlzweig was kind enough to supply us with samples of trigonelline and nicotinuric acid, and Dr. Eric G. Ball with the sample of diphosphopyridine nucleotide; the vitamin B₆ hydrochloride was furnished by Merck and Company.

nicotinuric acid
diphosphopyridine nucleotide
vitamin B₆ hydrochloride
dinicotinic acid
alpha picoline
alpha picoline methiodide
beta picoline
5,6-dichlor-nicotinic acid
trigonelline
5-amino-nicotinic acid
3,5-diamino-2,6-dimethyl pyridine
2,6-dimethyl pyridine
2,6-dimethyl pyridine hydrochloride
2,6-dimethyl dinicotinic acid (K salt)
diurethyl lutidine (3,5-diurethyl-2,6-dimethyl pyridine)
pyridine 2,3,5,6-tetracarboxylic acid
2,4,6-trimethyl dinicotinic acid (K salt)
1,4-dihydro-, 3,5-dicarbethoxy-, 2,4,6-trimethyl pyridine
1,4-dihydro-, 3,5-dicarbethoxy-, 2,6-dimethyl pyridine
3,5-dicarbethoxy-, 2,6-dimethyl pyridine
3,5-dicarbethoxy-, 2,4,6-trimethyl pyridine
quinoxaline 2-3-dicarboxylic acid
pyrazine monocarboxylic acid
pyrazine 2,3-dicarboxylic acid
2-methyl, 3-hydroxy-quinoxaline

Most of these compounds gave negative results. Both compounds containing a 5-amino substitution in the pyridine ring gave a fluorescence with an indigo blue tint resembling that of thiochrome, rather than that of the unknown; alkali was not needed to bring this out. Both compounds containing the 1,4 dihydro-, 3,5 dicarbethoxy substitutions gave a strong fluorescence, the color in both instances showing distinct differences from the unknown—the dimethyl compound giving a deep blue fluorescence and the trimethyl a violet emission; fluorescence appeared without the addition of alkali. The 3,5 dicarbethoxy dimethyl and trimethyl pyridines gave, also without alkali addition, a bluish fluorescence somewhat resembling the unknown, but the spectra given by these solutions were not identical with that of the fluorescent urinary extracts, from which it would appear that these compounds were not identical with the unknown. Diphosphopyridine nucleotide showed a blue fluorescence similar to that of the unknown, which likewise developed only after the addition of alkali. The fluorescent spectrum, however, showed marked differences from the unknown. Since the fluorescent spectrum of diphosphopyridine nucleotide has not, so far as we are aware, been studied, it is reproduced herewith. (Fig. 1.)

None of the compounds tested could, therefore, be identified with

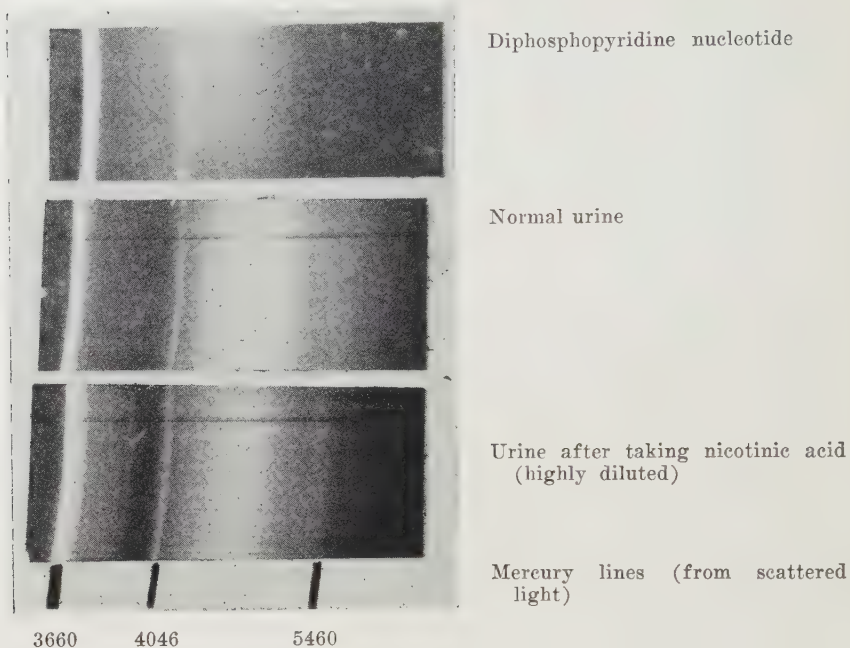


FIG. 1.
Photographs of Fluorescent Spectra.

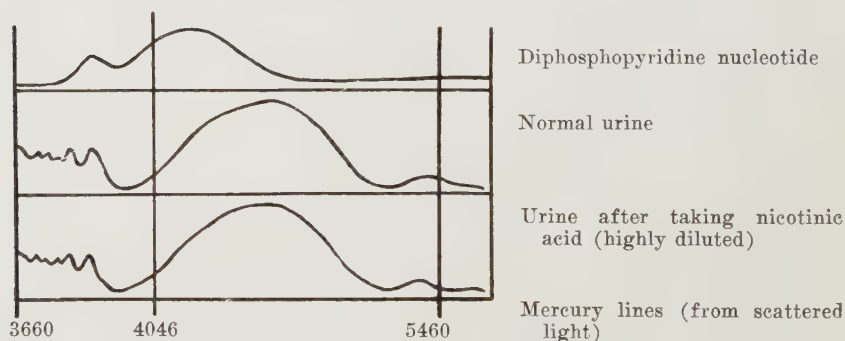


FIG. 2.
Spectrograms.

the unknown. An attempt was made to produce the unknown substance from nicotinic acid and from nicotinic acid amide *in vitro* by incubating normal urine with these compounds for 24 hours at 37° C. This procedure failed to increase the content of the unknown fluorescent material. The possibility that the unknown material might be a porphyrin was considered; however, spectroscopic examination of the KCl eluate failed to confirm this.

Photographs of the fluorescent spectrum were made with eluates

of normal urine and urine after nicotinic acid administration, the eluates being treated as before with alkali and extracted with butyl alcohol. The fluorescent spectra obtained, which are reproduced herewith, indicate that the substance present in small amount in normal urine is identical with that obtained in larger quantity after the ingestion of nicotinic acid. The photographs were taken with a small quartz spectrograph. The mercury lines appear faintly superimposed on the spectrum as the result of Rayleigh scattering of the solvent.

A number of observations were made upon the stability and differential solubility of the unknown substance. Boiling destroys the substance, more rapidly in the presence of alkali. At room temperatures it is destroyed slowly in alkaline solution and slowly by $K_3Fe(CN)_6$. The fluorescence of the butyl alcohol extract is readily destroyed by exposure to sunlight, but not by ultraviolet light passed through a Wood filter. It is readily extracted from aqueous solution by butyl or isobutyl alcohol, but not by amyl alcohol, octyl alcohol, chlorobenzene, benzene or chloroform.

The probability that this substance represents an excretion product of nicotinic acid suggests that its measurement may prove of value in states of nicotinic acid deficiency in man; one might anticipate finding a low value with subnormal excretion following a test dose of nicotinic acid. Up to the present we have had no opportunity of testing patients with nicotinic acid deficiency. Through the courtesy of Dr. C. A. Elvehjem we have tested the urine of a dog with black tongue before and after treatment. We were, however, unable to identify the unknown fluorescent material in the urine of this animal either before or after treatment with nicotinic acid, which suggests that the dog may conjugate this substance by a different mechanism from that which occurs in man.

In the hope that others may have an opportunity to apply this procedure to pellagrins, it is given in detail:

To 20 cc urine are added 10 g permutit ("Decalso" 30 mesh) in a small separatory funnel; this is shaken for 15 minutes gently. The permutit is then washed with 5 portions (30 cc each) of distilled water, the washings being discarded. The permutit is then dried by suction. Ten cc of a 25% KCl solution are then added and the funnel is shaken well for 15 minutes. The fluid is then allowed to drain from the permutit drop by drop the last portion being expelled by air. The permutit is washed with 2 to 4 cc of KCl solution which is added to the eluate previously collected. The eluate is then divided into two equal portions, to one of which one

cc of 15% NaOH is added. Both samples are then shaken immediately with 13 cc butyl alcohol for 3 minutes. The mixture is then centrifuged to separate the butyl alcohol layer and this is treated with anhydrous Na_2SO_4 to remove traces of water, and is allowed to stand in the dark for 20 minutes. Fluorescence is then determined in a Pfaltz and Bauer fluorophotometer, the source of light for which is a mercury vapor bulb (General Electric, type H3-85 watts) shielded by a Wood filter (Jena UG-2), the emitted fluorescence being measured after the interposition of a double filter of bright bluish green (Jena BG-14) and bright yellow (Jena GG-3). The difference in fluorescence between the sample treated with alkali and that not so treated represents the fluorescence of the unknown compound. Comparative quantitative measurements can be obtained by comparing the fluorescence with that of a quinine sulfate solution containing 10 to 25 μg % in 0.1 normal H_2SO_4 . The daily output of the unknown substance in urine of normal adults gives a fluorescence corresponding roughly to 100 μg of quinine sulfate. After the ingestion of 50 mg of nicotinic acid, the concentration in urine reaches 8 to 10 times its previous value, during the first 4 hours.

11464 P

A Simple Method of Preparing Dried Serum Proteins for Therapeutic Use.

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Attempts to preserve serum proteins in a dry form for therapeutic use have been made for a number of years. Flosdorf and Mudd's¹ method of preparing "lyophile" serum is for the most part satisfactory, but requires complicated and expensive special apparatus which was not at our disposal when we began to work with dried serum proteins. We therefore found it necessary to find or devise some method which could be carried out with ordinary laboratory apparatus. After preliminary trials with several other methods, we found the method devised by Hartley² for the preparation of dry and lipid-free immune sera to be most suitable,

¹ Flosdorf, E. W., and Mudd, S., *J. Immunol.*, 1935, **29**, 389.

² Hartley, P., *Brit. J. Exp. Path.*, 1925, **6**, 181.

and we have employed it, with slight modifications of the original method, since 1937.

A mixture consisting of 7 volumes of 95% alcohol and 3 volumes of redistilled absolute ether is cooled to -20°C or -18°C . To this mixture 1 volume of serum, previously cooled to 4°C , is added drop by drop and very slowly, the contents of the container being vigorously stirred for the entire time. After all the serum has been added, the flask is well shaken and allowed to stand for 2 hours.

The resulting fine white precipitate of serum proteins is filtered off on a Büchner funnel and is washed repeatedly with absolute ether cooled to -20°C . Since as much alcohol as possible should be removed, it is desirable, whenever practical, to use 20 volumes of absolute ether for the washings at this stage. The filter paper with the protein is placed over sulphuric acid in a vacuum desiccator, which is then evacuated to remove the remaining ether. Solid carbon dioxide is used to cool the precipitating and washing fluids.

When this procedure was carried out with small amounts of serum (10-20 cc), the protein preparation obtained was in the form of a coarse white powder, which was slowly soluble. A solution of this preparation was perfectly clear and resembled the original serum in appearance. Clear solutions can still be obtained from the dried preparations made 2 years ago.

Three experiments were carried out to test the physiologic properties of dried proteins prepared by this method from dog serum. (1.) Ten gram portions of dried serum were dissolved in physiologic saline solution by prolonged stirring, after which the solution was centrifugalized for 30 minutes at about 3,000 rpm to remove any undissolved protein present. It was then filtered through a Seitz bacteriological filter and the protein content was checked by the colorimetric method of Johnston and Gibson.³ Twenty cc of the solution (2 g of protein) were injected intravenously into a dog after a preliminary temperature observation had been recorded. No alterations in temperature were observed in observations made at 15-minute intervals for several hours after the injection. (2.) The experiment was repeated with a different protein solution on another dog without ill effects. (3.) A third dog was given 50 cc of solution (5 g of protein) representing a combination of 2 other protein preparations. A transitory temperature elevation of 1°C was observed.

Experiments were then carried out with human serum proteins of appropriate blood type, which were dried, dissolved, Seitz-filtered,

³ Johnston, G. W., and Gibson, R. B., *Am. J. Clin. Path. Tech. Suppl.*, 1938, 8, 22.

tested for sterility, and injected into a dog. When no reaction was observed in the animal, 40 cc of the same solution (4 g of protein) was injected intravenously into a patient. There was no apparent reaction.

When serum was used in 500-700 cc portions, some difficulty was encountered in the preparation of dried serum proteins due to denaturation of the proteins. The cause of the denaturation is probably the difficulty of stirring the larger amount of precipitating fluid adequately, as well as difficulties in filtration. If filtration is not accomplished very rapidly, the alcohol in the precipitating fluid tends to become warm enough to denature the serum proteins. These difficulties have not yet been entirely overcome.

Summary. The method devised by Hartley for the preparation of dry and lipid-free immune sera has been adapted to the preparation of dried serum proteins in quantity. In spite of some technical difficulties, the serum proteins prepared by this method have been injected into dogs and into a single human subject without serious reactions.

11465

Resistance of Human Spermatozoa *in vitro* to Sulfanilamide and Sulfapyridine.

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Jaubert and Motz¹ studied the effect of sulfanilamide on spermatogenesis in 23 men suffering from gonorrhea. They noted a reduction in both the number and vitality of the spermatozoa, with their complete immobilization in some instances. Marion, Barbellion, and Torres,² observed that small oral doses of sulfanilamide caused a decrease in the number and motility, and an increase in the abnormal forms, in the spermatozoa of 69% of their patients. Vigoni³ found the same changes in men treated by urethral irrigation with sulfanilamide, 2 of his patients actually developing azoospermia.

* Fellow, National Committee on Maternal Health.

¹ Jaubert, A., and Motz, C., *Presse méd.*, 1938, **46**, 237.

² Marion, Barbellion and Torres, *Bull. Soc. fran. d'urol.*, May 16, 1938.

³ Vigoni, M., *J. Belge d'urol.*, 1938, **11**, 375.

On the other hand, Heckel and Hori⁴ found no noteworthy effects on spermatogenesis in 11 normal patients given sulfanilamide. Levaditi and Vaisman⁵, as well as Palazzoli, Nitti, Bonet, and Levinson,⁶ observed little effect from sulfanilamide on spermatogenesis in experiments with mice, rats and rabbits.

Since all of the work referred to was carried out *in vivo*, and as far as the author is aware, no experiments have been made on the *in vitro* effect of sulfanilamide on spermatozoa, the following studies were conducted on the effect of sulfanilamide and sulfapyridine as well.

Materials and Methods: The seminal specimens studied in these experiments were of varying freshness. They were furnished by 20 donors ranging from 17 to 51 years. Approximately 0.01 ml of semen was thoroughly mixed with 1 ml of Baker's⁷ fluid† containing either sulfanilamide or sulfapyridine in concentrations of 5 to 160 mg %; the specimens thus mixed were kept both at 22°C (approximately) and body temperature. Control tests were conducted with the same amount of semen in Baker's fluid alone, also kept at the 2 temperatures. Sulfanilamide and sulfapyridine were also added to 1 ml of undiluted semen in a number of experiments, in an amount sufficient to give maximum concentration at the respective temperatures, undiluted semen alone being used as a control. Observations were made at frequent intervals, particularly toward the end of an experiment, so that the time of cessation of all movement could be accurately ascertained. Depression slides were used and while observations were not being made, they were kept in closed petri dishes, to prevent evaporation. The results obtained are presented in Tables I, II.

Results: The tables demonstrate no correlation between the age of the donor and the length of survival of the spermatozoa. There was marked individual variation in the duration of motility among different specimens, but the average time of survival at room temperature (9.4 hours) was considerably longer than at body temperature (3.5 hours). It can also be seen that the survival time of spermatozoa in various concentrations of sulfanilamide and sul-

⁴ Heckel, N. J., and Hori, C. G., *Am. J. Med. Sci.*, 1939, **198**, 347.

⁵ Levaditi, C., and Vaisman, A., *Compt. rend. Soc. de biol.*, 1938, **128**, 352.

⁶ Palazzoli, M., Nitti, F., Bonet, D., and Levinson, M., *Compt. rend. Soc. de biol.*, 1938, **128**, 261.

⁷ Baker, J. R., *J. Hyg.*, 1931, **31**, 309.

† The composition of Baker's fluid is as follows: water, 1000 ml; glucose, 30.9 g; Na₂HPO₄ · 12 H₂O, 6.0 g; NaCl, 2.0 g; KH₂PO₄, 0.1 g.

fapyridine at both room and body temperatures was essentially the same as that of the respective controls. Neither the age of the specimen nor donor altered either susceptibility or resistance to these drugs. The results were equally negative if the sulfanilamide and sulfapyridine were added to Baker's solution plus semen or to undiluted semen.

Since an *in vitro* concentration of as much as 160 mg % was used, these results gain added significance when it is recalled that the maximum tissue fluid concentration of sulfanilamide achieved clinically is about 15 mg % and that of sulfapyridine about 10 mg %.

Summary: *In vitro* concentrations of sulfanilamide and sulfapyridine well above the tissue concentration achieved by therapeutic doses, do not affect the survival or activity of human spermatozoa.

11466

A New *Salmonella* Type Isolated from Apparently Normal Hogs.*

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In a study of the mesenteric lymph glands of apparently normal hogs in Uruguay, Hormaeche and Salsamendi¹ isolated numerous *Salmonella* types. In a repetition of this work Rubin² found that *Salmonella* strains could be isolated frequently from the mesenteric lymph glands of apparently normal hogs slaughtered at an abattoir in Kentucky. The purpose of the present paper is to describe a hitherto unrecognized *Salmonella* type encountered in these hogs. The organism is designated as *Salmonella lexington*.

Methods—Two mesenteric lymph glands were removed from each hog after the internal organs had been inspected. Lymph glands from 25 hogs were placed in a sterile container, taken to the labora-

* The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

¹ Hormaeche, E., and Salsamendi, R., *Arch. Urug. Med., Cir. y Espec.*, 1939, **14**, 375.

² Rubin, H. L., unpublished data.

tory and thoroughly ground with sand in a sterile mortar. To the ground mass was added 30 cc of sterile saline and 1 cc of the mixture was placed in each of 3 tubes of the tetrathionate enrichment medium of Kauffmann.³ After overnight incubation the enrichment medium was plated on brilliant green agar. *Salmonella*-like colonies which developed on the plates were examined serologically and their position in the Kauffmann-White classification determined. The methods used in the serological tests were those employed by Edwards.⁴

Results—In the particular lot of glands from which the new type was isolated 3 *Salmonella* species were found. In addition to *S. lexington*, *S. derby* and *S. bareilly* were also present.

The microorganism was a motile rod which possessed the usual biochemical and tinctoral properties attributed to the genus *Salmonella*. It produced acid and gas from arabinose, dulcitol, glucose, inositol, mannitol, rhamnose, sorbitol, trehalose and xylose. Adonitol, lactose, salicin and sucrose were not attacked. Hydrogen sulfide was produced from 2% peptone water.

Examination of the somatic antigens of *S. lexington* revealed that it belonged to group E of the Kauffmann-White classification. Alcoholized suspensions were agglutinated to the titre of *S. nyborg* antiserum and absorption of the serum with *S. lexington* removed all somatic agglutinins for the homologous strain. The somatic antigens of *S. lexington* are III X XXVI

When the flagellar antigens of *S. lexington* were examined it was found that the organism was diphasic and displayed specific-non-specific phase variation. The specific phase was flocculated to the titre of serum derived from the alpha phase of *S. glostrup*, but was not affected by serums derived from the other antigens represented in the Kauffmann-White classification. Absorption of *S. glostrup* alpha serum with the specific phase of *S. lexington* removed all flocculating agglutinins for the homologous strain. The antigen of the specific phase of the organism is z_{10} .

The nonspecific phase of *S. lexington* was agglutinated by serums derived from all the nonspecific phases of the Kauffmann-White schema. It was then tested with absorbed serums containing the factors 2, 3, 5, 6 and 7, respectively. Agglutination occurred only in the presence of factors 3 and 5. When antiserum derived from the nonspecific phase of *S. choleraesuis* was absorbed with the nonspecific phase of *S. lexington* residual agglutinins amounting to less than

³ Kauffmann, F., *Z. f. Hyg.*, 1935, **117**, 26.

⁴ Edwards, P. R., *J. Bact.*, 1936, **32**, 259.

2% of the original titre were left for the homologous strain. The nonspecific antigens of *S. lexington* are 1,5...

Summary: A new *Salmonella* type, *Salmonella lexington*, is described. It was isolated from the mesenteric lymph glands of apparently normal hogs. The organism is represented by the antigenic formula III X XXVI:z₁₀:1,5...

11467 P

Mineral Distribution in Some Nerve Cells and Fibers.*

GORDON H. SCOTT

From the Department of Anatomy, Washington University School of Medicine, St. Louis.

It is known from examination of many types of tissue that Mg and Ca, as revealed by the electron microscope, is located in areas which show white ash following microincineration. In nerve tissue certain difficulties have hampered a direct study of Ca and Mg by means of the electron microscope. Some of the findings in incinerated sections of frog sciatic and sympathetic ganglia are believed to be of significance although we have not been able to identify the salts as clearly as is desirable.

When sections of frozen and dehydrated (Scott and Packer¹) frog sciatic are carefully incinerated and examined by dark field (Scott²) the large myelinated fibers at the periphery of the nerve leave residues of white ash probably consisting largely of Ca and Mg. The ash is clearly the remains of the myelin sheath as it corresponds almost exactly with stained preparations of the same nerve taken a few levels either above or below. The point to emphasize, however, is that there is no visible residue of any sort in the tissue spaces surrounding the nerve fibers.

In sharp contrast to plentiful mineral in the nerve fibers and little if any in the tissue space is the picture obtained when sympathetic ganglia are incinerated following the same treatment. The sympathetic ganglion cells are recognizable by their residue. Nuclear, nucleolar and Nissl substance ash is dense and of the variety associated with the presence of Ca and Mg. There is as a general rule

* Aided by a grant from the Rockefeller Foundation.

1 Scott and Packer, *Anat. Rec.*, 1939, **74**, 17, 31.

2 Scott, G. H., *Am. J. Anat.*, 1933, **53**, 243.

a wide band, varying from an eighth to a sixth of the cell diameter, of dense white ash concentrated at the periphery of the cell. The tissue spaces immediately about the ganglion cells are filled with mineral residue not unlike, in quality and quantity, that seen in the neurones.

It seems evident from these observations that like conditions of surrounding medium do not obtain in nerve fibers of the frog sciatic and in the cells of the sympathetic ganglia of the same animal.

11468

Direct Action of Estrone on the Mammary Gland.

WM. R. LYONS AND Y. SAKO

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Within the past decade, the hormonal control of mammary growth has been greatly clarified due to the availability of: (1) pure estrogenic compounds which by themselves cause growth of the nipple and ducts as well as a slight degree of alveolar development, (2) progesterone which in proper combination with estrone (or other estrins) causes complete lobular development such as occurs in pregnancy, and (3) mammotropin, the pituitary lactogenic hormone which causes functional growth and lactation in the alveoli or milk-secreting units in glands developed by estrin or estrin-progestin.

According to some investigators, the mammary glands of hypophysectomized animals do not respond as well (or at all) to estrin, and for this reason, Turner and co-workers^{1, 2} have proposed that the sex hormones merely stimulate the pituitary which in turn secretes 2 mammary-stimulating substances, mammogen I which induces duct development and mammogen II causing lobule-alveolar growth. These investigators have extracted a fat-soluble substance from the pituitary, which they identify with mammogen I because it causes mammary duct development in male and female mice. They³ also obtained lobule-alveolar growth in castrated female mice by injecting fresh pituitaries from pregnant cattle. Because this material caused alveolar proliferation over and above the duct development induced by their extracts these investigators have postulated a second[†] mammogen.

¹ Lewis, A. A., Turner, C. W., and Gomez, E. T., *Endocrinol.*, 1939, **24**, 157.

² Lewis, A. A., and Turner, C. W., *Mo. Agr. Exp. Sta. Bul.* 182, 1939.

³ Mixner, J. P., Lewis, A. A., and Turner, C. W., *Anat. Rec.*, 1940, Suppl., **76**, 43.

The findings of the Turner laboratory might be interpreted on the basis of earlier investigations^{4, 5} showing that estrin and progestin could be extracted from the pituitary, but it has been claimed that mammogen I, although weakly estrogenic, has mammary-stimulating potency greater than the purified estrins now available.² While these findings are in themselves interesting and significant, they in no way support the contention of the Missouri group that the ovarian hormones act only indirectly upon the mammary gland. In fact, suggestive evidence to the contrary has been at hand for some time now.⁶ In each of 3 women, MacBryde obtained good development in the breast rubbed with estradiol in ointment (25,000 I.U., daily for 2 weeks) while the contra-lateral breast rubbed only with the ointment base showed much less growth. We have carried out in male rabbits a similar experiment designed to sustain the theory of the direct action of estrin, with the advantage over the human work reported, of being able to know more precisely the histologic nature of the growth response.

Experimental. Preliminary experiments were carried out in which the approximate amount of estrin was determined that could be expected to act only on the gland locally treated, and also the amounts of hormone that would be sufficient to allow for some absorption and therefore remote action on the glands not treated directly. And then, 2 groups of three, 2-month-old male rabbits, weighing approximately 2 kg were treated with 2 distinct levels representing threshold and sub-threshold doses of estrone.

The estrone* in sesame oil was rubbed into the skin immediately around the nipples on the left side, while sesame oil alone was administered in like manner to the nipple regions on the right side. Three of the rabbits had an accessory or ninth nipple and this was left untouched, thus serving as a further control. A single drop of oil (approximately 0.03 cc) was applied with a medicine dropper directly to the nipple. The oil usually spread out over a skin area not greater than 3.0 cm and this was rubbed with the end of the finger and gently massaged between the thumb and forefinger. The 3 rabbits in Group 1 were treated with an estrone preparation containing 100 I. U. per cc or 3 I.U. per drop, while the preparation used in Group 2 contained 10 I.U. per cc or 0.3 I.U. per drop. In all 25 drops were administered to each gland (Monday through

⁴ Brouha, L., and Simmonet, H., *C. R. Soc. de Biol.*, 1927, **96**, 1275.

⁵ Callow, R. K., and Parkes, A. S., *J. Physiol.*, 1936, **87**, 28 P.

⁶ MacBryde, C. M., *J. A. M. A.*, 1937, **112**, 1043 (has earlier references).

* Kindly supplied by Dr. Oliver Kamm of Parke, Davis and Company.

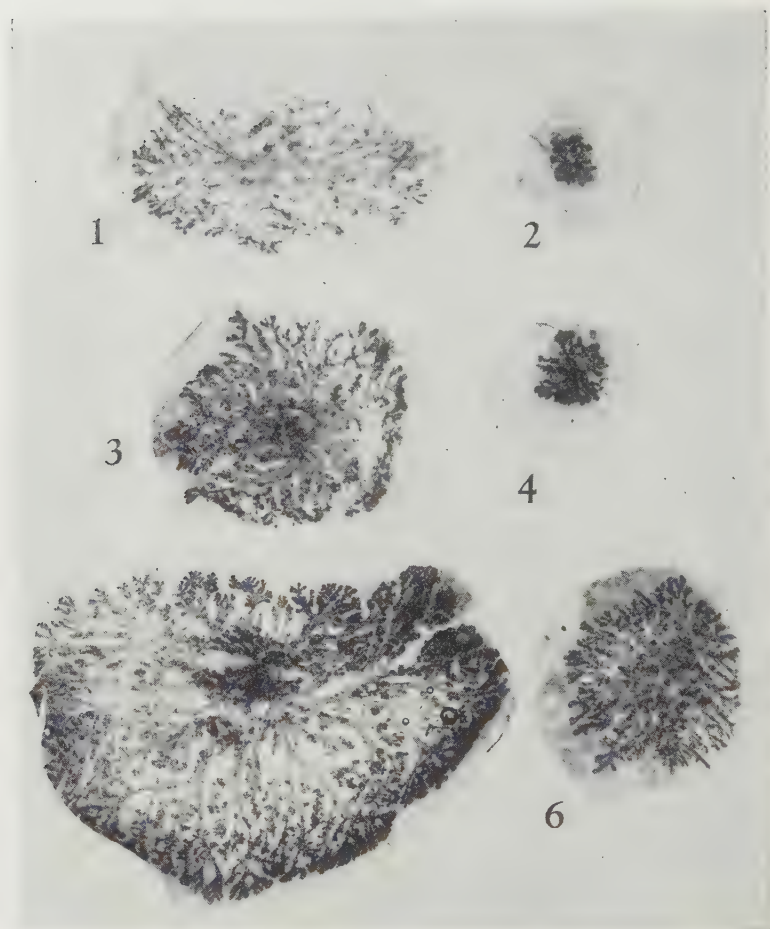


FIG. 1. Typical left mammary spread from male rabbit 1 of group 2. A drop of sesame oil containing approximately 3 I.U. of estrone was rubbed into the skin overlying this gland daily for 25 days. Note evidence of extensive duct growth. All figures $\times 1.5$.

FIG. 2. Typical right mammary spread from male rabbit of group 2. Sesame oil only was rubbed into skin. Gland is rudimentary and resembles that of untreated, normal male rabbits.

FIG. 3. Typical left mammary spread from male rabbit 2 of group 2. A drop of sesame oil containing approximately 3 I.U. of estrone was rubbed into the skin overlying this gland daily for 25 days. Note evidence of extensive duct growth.

FIG. 4. Typical right mammary spread from male rabbit 2 of group 2. Sesame oil only was rubbed into skin. Gland is rudimentary and resembles that of untreated, normal male rabbits.

FIG. 5. Typical left mammary spread from male rabbit 3 of group 2. A drop of sesame oil containing approximately 3 I.U. of estrone was rubbed into the skin overlying this gland daily for 25 days. This gland shows more extensive duct growth as well as some alveolar development.

FIG. 6. Typical right mammary spread from rabbit 3 of group 2. Although only sesame oil was rubbed into skin overlying this gland, enough estrone was absorbed on the left side to circulate and cause the duct growth shown here.

Friday for 5 weeks), making a total of approximately 75 I.U. per gland or 300 I.U. per animal (4 glands) in the first group, and 7.5 I.U. per gland or 30 I.U. per rabbit in the second group.

Results. It became apparent after 2 to 3 weeks of treatment that the nipples receiving the 3 I.U. estrone per dose were growing at a faster rate than the controls as well as those receiving the 0.3 I.U. estrone. At the end of the fifth week, the estrone-treated nipples of both groups were all considerably larger than their contra-lateral controls, but the control nipples of Group 1 were larger than the control nipples of Group 2 indicating that some of the estrone was being absorbed and probably circulated to the contra-lateral glands. At necropsy spreads were made of 53 mammary glands. These were stained *in toto* with alum carmine and cleared in methyl salicylate. None of the glands taken from the animals in Group 1 showed development greater than that seen in normal males of the New Zealand White strain used. Two of the 3 rabbits treated with higher doses of estrone showed duct growth only in the glands rubbed with the hormone, Figs. 1 and 3, the sesame control and untreated glands all being within the normal limits (Fig. 2 and 4). The third animal of this group was more responsive to estrone for not only did its estrone-treated glands (Fig. 5) show better development than the estrone-treated glands of the other rabbits, but its control glands (Fig. 6) also showed considerable development. As in the case of the nipples in both groups, this indicated that some estrone was being absorbed and circulated. However, the striking difference between the hormone-treated and control glands provides just as good proof of the direct action of estrone as the all-or-none effect observed in the other animals.

Ruinen⁷ had attempted this same experiment in 1932, but used a much larger dose of hormone (100 units daily of menformon). With such an excess, he obtained equal development in the mammary glands of both sides. As the author admits, his findings still did not preclude the possibility of a direct action of estrin on the mammary gland.

Summary. The proper dose of estrone in oil rubbed into the skin over the rudimentary mammary glands of young male rabbits caused growth only of those glands, and not of the control glands treated with oil. Such evidence supports the view that estrogenic substances are directly mammary-stimulating.

⁷ Ruinen, F. H., *Acta Brev. Neerl.*, 1932, **2**, 161.

Maintenance of Pregnancy in Castrate Rats by Means of Progesterone.*

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Corner and Allen's¹ early successful attempts to maintain pregnancy in rabbits castrated shortly after mating could not be duplicated by Allen and Heckel² using crystalline progesterone, unless the castration occurred after implantation.³ Pincus and Werthessen,⁴ however, maintained gestation in one out of three rabbits when sufficient crystalline progesterone was used, while in short time experiments Courier and Kehl⁵ maintained pregnancy in rabbits with progesterone, but in only one rabbit was castration done before implantation. Courier and Jost⁶ using large amounts of pregnenolone caused rabbits to implant normally. Maintenance of pregnancy has been successfully accomplished in other animals (rats,^{7, 8} ground squirrels,⁸ mice⁹ and hamsters¹⁰) using progestin,⁸ progesterone^{9, 10} or androgens,⁷ when castration was done after implantation.

Previously¹¹ it was shown that placentomata indistinguishable from those formed in normal pseudopregnant rats could be produced in the castrate rat by means of progesterone alone. In the present study it will be shown that normal implantation as well as continued gestation will occur in the castrate rat when sufficient progesterone is administered.

A total of 26 pregnant rats were used, 22 of which were castrated on the 4th, and 4 on the 10th day of pregnancy. All rats, except

* This work was supported by the Wisconsin Alumni Research Foundation. Assistance was also furnished by the personnel of the WPA Official Project No. 65-1-53-2349.

¹ Corner and Allen, *Proc. Soc. Exp. Biol. and Med.*, 1930, **27**, 403.

² Allen and Heckel, *Science*, 1937, **86**, 409.

³ Allen and Heckel, *Am. J. Physiol.*, 1939, **125**, 31.

⁴ Pincus and Werthessen, *Am. J. Physiol.*, 1938, **124**, 484.

⁵ Courier and Kehl, *Comptes rendus Soc. de Biol.*, 1938, **128**, 188.

⁶ Courier and Jost, *ibid.*, 1939, **130**, 1162.

⁷ Greene and Burrill, *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 585.

⁸ Johnson and Challans, *Endocrinol.*, 1932, **16**, 278.

⁹ Robson, *J. Physiol.*, 1938, **92**, 371.

¹⁰ Klein, *Proc. Roy. Soc., B*, 1938, **125**, 348.

¹¹ Rothchild, Meyer and Spielman, *Am. J. Physiol.*, 1940, **128**, 213.

those in the first 2 experiments, were sterilized by ovario-salpingectomy of one horn of the uterus on the day after coitus, and the sterile horn traumatized by needle punctures through the anti-mesometrial wall on the 4th day of pregnancy. This permitted a concomitant study of the effect of the hormone treatment on the formation of placentomata. Hormone treatment was started on the day of complete castration, and continued daily up to and including the 20th day of pregnancy. All rats were autopsied on the 21st day, except in those cases where, by means of laparotomies performed between the 9th and 17th day, it was seen that pregnancy had terminated. Hormone treatment consisted of progesterone[†] alone, or in combination with estradiol,[‡] with corn oil as the solvent.

From Table 1 it can be seen that negative results were obtained with amounts of progesterone of less than 1 Rb.U. per day. The smaller doses of progesterone, however, permitted the formation of implantation sites, which in the rats of Exp. I did not persist beyond the 13th day, but in 2 of the 4 rats of Exp. II persisted normally to

TABLE I.

Exp. No.	Daily hormone treatment		Rats treated	Rats maint. in pregn. to 21st day		
	Progest.*	Estradiol, γ		No.	Implant. Sites	Term Fetuses
I	.3	.03	4	0	—	—
II	.6	.03	4	0	—	—
III	1.0	—	5	2	5 5	1 1
IV	1.0	.15	3	1	2	1
V†	2.0	—	4	3	4 3 4	3 2 4
VI	2.0	—	5	4	2 3 4 4	1 2 3 1

* Rabbit units of progesterone.

† These rats were castrated on the 10th day of pregnancy.

‡ The source of progesterone was a non-crystalline preparation containing 10%-50% of progesterone (Corner-Allen Rabbit Units) and was made from cholesterol by the Spielman process. (Spielman and Meyer, *J. A. C. S.*, 1939, **61**, 893.)

† The estradiol was supplied through the courtesy of the Schering Corporation, Bloomfield, N. J.

the 17th day. Implantation proceeded normally, as far as could be determined macroscopically, in all the remaining experiments, and at least some of the rats in each experiment carried living young to the 21st day. The percentage of completely maintained rats, as well as the ratio of living young to total number of implantations, increased with increase in the amount of progesterone administered.

The placentomata which formed in the sterile horn of the uteri of all the rats of Exp. III-VI were larger in every case than the implantation sites in the pregnant horn. This would indicate that the formation of decidual tissue in the rat is dependent, not only upon the size of the progesterone dose,¹¹ but possibly upon the strength of the traumatic stimulus as well, since it is most likely that the trauma of the uterine epithelium produced by the implanting egg is not of the same order of magnitude as that used in the artificial production of placentomata.

The possibility that contaminants in the progesterone preparation might have influenced the results must be admitted, but we do not believe that they played an important part. In other experiments,^{11, 12} using the same type of preparations, we found no quantitative or qualitative differences between the non-crystalline and crystalline progesterones.

Summary. Rats castrated on the 4th day of pregnancy were maintained in pregnancy until the 21st day with daily doses of progesterone of 1 or 2 Rb.U.

11470 P

Precipitation Pattern of Serum Proteins in Phenylpyruvic Oligophrenia.

ALBERT A. KONDRITZER (Introduced by Warren M. Sperry)

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Jervis, *et al.*,¹ have shown that the serum of phenylpyruvic oligophrenic individuals contains an abnormal amount of phenylalanine. In view of the recognized effects of small amounts of amino acids on the molecular dispersion of the proteins² it seemed possible that

¹² Rothchild and Meyer, *Anat. Rec.*, 1939, **75**, suppl. 1, 71.

¹ Jervis, G. A., Block, R. J., Bolling, D., and Kanze, E., in press.

² Tiselius, A., *Ann. Rev. Biochem.*, 1939, **8**, 155.

abnormalities in the state of the serum proteins might occur in this disease. Perlzweig, Kondritzer and Bruch³ found that in pathological conditions fractional precipitation of the serum proteins with gradually increasing quantities of potassium phosphate at pH 6.5-6.8, according to Butler and Montgomery,⁴ frequently reveals significant changes which are not brought out by the conventional methods for the determination of albumins and globulins by precipitation with an arbitrarily fixed concentration of a neutral salt.

In the present investigation the fractional precipitation procedure was applied to the serum proteins of 8 physically healthy patients in whom a diagnosis of phenylpyruvic oligophrenia had been made, and 8 healthy persons as controls. Nineteen portions of each sample of serum were precipitated with an equimolar KH_2PO_4 - K_2HPO_4 buffer which ranged in molality from 1.2 to 3.0 mols in 0.1 mol increments. In 5 of the patients and 2 of the normal subjects 0.5 cc portions of freshly centrifuged serum were added to 15 cc portions of buffer (serum dilution 1:31). In the remaining experiments in which less serum was available the serum was diluted with an equal volume of physiological saline; then 0.5 cc portions were added to 10 cc portions of the buffer solutions (serum dilution 1:42). After standing overnight at room temperature the precipitate was filtered off and the total nitrogen in solution was determined (microkjeldahl) on a suitable aliquot. The value was corrected for the N.P.N., determined on a trichloroacetic acid filtrate of the original serum, and the percentage of the total protein remaining in solution at each molality of phosphate was calculated. The data obtained in experiments in which the dilution of the serum was 1:42 were calculated to a dilution of 1:31 and combined with the data at the latter dilution. The average values obtained in the 8 normal and the 8 phenylpyruvic sera were plotted against the concentrations of the phosphate solutions (Fig. 1).

A small but consistent difference between the two solubility-precipitation curves in the middle range of phosphate concentration is apparent; more protein appears to have been precipitated from the sera of the phenylpyruvics than from those of the healthy controls. As the differences were small the data were subjected to rigorous statistical analysis with the kind assistance of Dr. Joseph Zubin. The methods which Fisher⁵ developed for analyzing small series of

³ Perlzweig, W. A., Kondritzer, A. A., and Bruch, E., *Proc. Am. Soc. Biol. Chem.*, 1938, **32**, xcii.

⁴ Butler, A. M., and Montgomery, H., *J. Biol. Chem.*, 1932, **99**, 173.

⁵ Fisher, R. A., *Statistical Methods for Research Workers*, 1934, Edinburgh and London.

data were applied in the modification of Snedecor.⁶ The F value obtained gives a measure of the probability that the difference between 2 means is significant. For averages obtained on 2 series of 8 determinations each the critical values of F those which could arise by chance not more than 1 to 5 times in 100, lie between 9.07 and 4.67 respectively. It will be seen from the chart that F values in this range were obtained for 3 phosphate concentrations between 2.0 and 2.3 mols per liter.* The chance that fortuitous differences of this degree of significance would occur at adjacent points is exceedingly small.

Each value for the percentage of the protein remaining in solu-

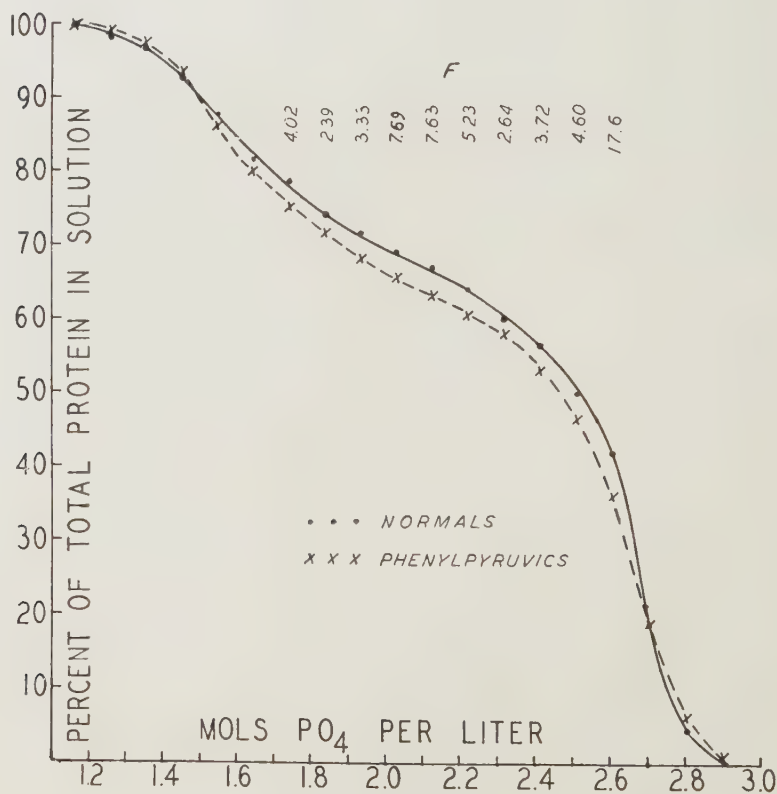


FIG. 1.

⁶ Snedecor, G. W., *Analysis of Variance*, 1394, Collegiate Press, Inc., Ames, Iowa.

* For this range of phosphate concentrations the effect of the difference in dilution (1:31 and 1:42) on the solubility of the serum proteins was shown during the statistical analysis to be of no significance.

tion was calculated from three nitrogen estimations (protein and N.P.N. in the original serum, and the total nitrogen of the filtrate). The chances for a cumulative error are, therefore, rather high. However, all determinations were carried out in the same apparatus under identical conditions, as nearly as they could be controlled, and hence the statistical treatment evaluated automatically the effect of errors arising in the analysis. Furthermore, errors in the determination of the protein and N.P.N. of the original serum would have a uniform effect throughout for each sample of serum and could not account for significant deviations in a portion of the curve.

It is concluded that there was a small but significant increase above normal in the globulin fraction of the serum proteins of the individuals with phenylpyruvic oligophrenia studied in this investigation. It is possible that the increase may have resulted from factors other than the particular pathology involved in this disease.[†]

11471

Response of Various Breeds of Rabbits to Hamilton and Schwartz Test for Parathyroid Secretion.

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In our earlier work¹ with the Hamilton and Schwartz² test for parathyroid hormone, we used 2 breeds of rabbits, raised by ourselves; a black and white Dutch strain and a gray Belgian strain, which included some albinos. All these rabbits gave a positive test when injected with 10 Hansen units of parathormone per kg. In a few instances injection with as little as 4 units per kg resulted in a positive reaction. Similar responses were given by hybrids of these 2 strains. (The H. and S. test depends upon the fact that successive feedings of CaCl_2 result in smaller and smaller rises in serum Ca, so that after the 3rd or 4th administration of 100 mg of Ca as CaCl_2 normal rabbits will show a rise of serum Ca of less than 1.2 mg per 100 cc, whereas if more parathyroid hormone than that normally circulating is present, a greater rise of serum Ca results, roughly proportional to the quantity of hormone administered.)

[†] The phenylpyruvic blood samples were obtained from inmates of Letchworth Village through the courtesy of Dr. Harry C. Storrs, Superintendent.

¹ Baumann, E. J., and Sprinson, D. B., *Am. J. Physiol.*, 1939, **125**, 741.

² Hamilton, B., and Schwartz, C., *J. Pharm. and Exp. Therap.*, 1932, **46**, 285.

When New Zealand white or chinchilla rabbits were used for this test, it was found they were less sensitive than the Dutch or Belgian breeds. They required an injection of 20 or 30 units of parathormone per kg to react positively. Six chinchillas and 4 New Zealand whites all gave negative reactions when injected with 10 units per kg. With a dose of 20 units per kg of parathyroid extract only 3 of 5 chinchillas and 1 of 3 New Zealand white rabbits reacted positively, while 2 of each of these strains gave positive reactions only with a dose of 30 units per kg.

It is advisable, therefore, to determine the sensitivity of rabbits to be used for the Hamilton and Schwartz test. The animals should be at least 5 months old and they should be kept on a diet whose Ca:P ratio is one or more for several days before use.

11472 P

Agent of Lymphogranuloma Venereum in the Lungs of Mice.

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It has been shown¹ that the agent of lymphogranuloma venereum readily initiates a fatal infection when introduced into the yolk-sac of the developing chicken embryo, in contradistinction to the well known low-grade character of the infection which results when the virus is placed on the chorio-allantois. In the former site the minute "granulocorpuscles"² which are believed to represent elementary bodies of the agent are found in enormous numbers. With this source of abundant virus at hand the possibilities of intranasal infection in mice were investigated, as has also been done recently by Schoen³ who employed virus propagated in the Ehrlich mouse sarcoma.

Two strains* of the lymphogranuloma venereum agent were

¹ Rake, G., McKee, C. M., and Shaffer, M. F., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 332.

² Miyagawa, Y., Mitamura, T., Yaoi, H., Ishii, N., Nakajima, H., Okanishi, J., Watanabe, S., and Sato, K., *Jap. J. Exp. Med.*, 1935, **13**, 733.

³ Schoen, R., *C. R. Acad. Sci.*, 1939, **208**, 772.

* One strain was obtained through the courtesy of Dr. Wm. L. Fleming, the School of Hygiene and Public Health, Johns Hopkins University. The second was obtained through the courtesy of Dr. Marion Howard, Department of Medicine, Yale University.

studied in Swiss mice weighing usually less than 10 g. Ground suspensions of yolk-sacs heavily infected with virus were diluted ten-fold with broth and centrifuged to throw down tissue fragments. 0.03 to 0.05 ml of supernatant was inoculated intranasally under light ether anesthesia. The mice which within 48 to 72 hours were manifestly ill with signs of marked respiratory involvement, were sacrificed; some died during this period. The former at autopsy showed, in one or more lobes, areas of semi-translucent gray-red consolidation varying in extent; the dead mice showed hemorrhagic consolidation of nearly all of the lung tissue. On microscopic examination of smears made by streaking a fragment of consolidated lung on a slide, fixing the film in methyl alcohol and staining with Giemsa stain, numerous elementary bodies could be seen lying free or within monocytic cells. In histological sections the picture was one of pneumonia, varying in degree but often very intense. This was both interstitial, with an accumulation of fluid and cells in the walls of the alveoli, and lobular with filling of the alveoli with fluid, monocytes and neutrophils. In the cytoplasm of certain cells, apparently the lining cells of the alveoli, there could be seen in most lungs large vacuoles filled with elementary bodies or larger inclusions both of which stained purple with Giemsa. In addition, clumps of elementary bodies were seen lying free in the alveoli and bronchioles. The microscopic changes during the development of the lung lesions will be described in greater detail elsewhere.

Serial intranasal passages in mice under light ether anesthesia were readily effected by sub-inoculation of broth suspension of affected lung tissue taken on the 2nd to 4th days after infection. With one strain 39 such lung-passages have been attained; with the other strain, 24 passages. Mice given intranasally 10^{-2} dilution of grossly diseased lung tissue usually died within 5 days with almost total pneumonic consolidation. Fatal illness was less frequent in animals receiving 10^{-3} dilution; many of these mice exhibited a transient malaise but eventually recovered. If sacrificed between the 3rd and 5th days, however, macroscopic lung lesions were found in most mice receiving 10^{-3} dilution. Lesions were not found in mice receiving 10^{-4} and 10^{-5} dilutions. Nevertheless, at the end of 4 days' infection virus was present in considerable amount in the lungs of mice receiving 10^{-4} dilution as could be demonstrated on serial intranasal passage, and it seems certain that multiplication had occurred.

The possibility that the lesions obtained in the lungs were due to organisms of the pleuro-pneumonia group or to infection with the latent virus of mouse pneumonia⁴ was excluded, in the first case by

cultures on suitable media and in the second, by cross-neutralization tests with anti-sera supplied through the courtesy of Dr. F. L. Horsfall.

In the case of both strains, inoculation of 1 ml of 10^{-7} and occasionally 10^{-8} dilution of consolidated lung tissue into the yolk-sacs of 5- or 6-day eggs incubated at 36° C sufficed to bring about death of the developing chicken embryos after several days' infection. The yolk-sacs of these eggs examined immediately after death were bacteriologically sterile but showed innumerable elementary bodies in Giemsa-stained smears. These findings not only confirm the microscopic observations as to the presence of large amounts of virus in the lungs of mice intranasally infected with lymphogranuloma venereum agent but also serve to reemphasize the delicacy of the yolk-sac technic as compared with all other methods at present available for the detection of the virus. The obvious usefulness of the pulmonary infection[†] of mice in immunological experimentation on lymphogranuloma venereum is being further explored.

11473

Complement Fixation Test in Lymphogranuloma Venereum.

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Although it is generally acknowledged that the cutaneous test with the Frei antigen is of great value in establishing the diagnosis of infection with the etiological agent of lymphogranuloma venereum, many workers have sought to devise other procedures which might be employed as corroborative evidence. The serological technic most widely explored in this connection has been complement fixation but, using a variety of antigens, most investigators have been unsuccessful in their attempts to demonstrate a specific reaction (for literature see¹). Nearly all the reports of positive findings are justly open to criticism on such grounds as inadequately detailed description of the method, lack of controls, or incomplete data on the results.

⁴ Horsfall, F. L., and Hahn, R. G., *J. Exp. Med.*, 1940, **71**, 391.

[†] Workers should bear in mind the possible hazards involved in the use of the intranasal technic where high concentrations of virus are concerned.

¹ Melczer, N., and Sipos, K., *Arch. f. Dermat. u. Syph.*, 1937, **176**, 176.

Since the quantitative relationships of the reagents are of paramount importance in serological tests it seemed likely that many of the failures might be due to the use of antigens of insufficient potency and that it might be worthwhile to reinvestigate the potentialities of the complement fixation test in this disease, with preparations containing higher concentrations of antigen than had hitherto been available. The following antigens have been employed: (a) the consolidated lungs of several mice, infected intranasally² with the agent of lymphogranuloma venereum, were pooled and ground with Pyrex fragments plus broth to a 10% suspension. This was freed of gross particles by centrifugation at 2000 RPM. The supernatant, termed "Lygranum" (M.L.) antigen, was stored at -32°C until needed, when it was thawed and further diluted with 0.85% saline to a final lung concentration of 1:100 or 1:150 for use in the test. Normal mouse lung suspension similarly prepared served as control. (b) Five- or 6-day eggs, inoculated via the yolk-sac³ with lymphogranuloma venereum agent, were incubated at 36°C until the death of the chicken embryo. Immediately thereafter the bacteriologically sterile yolk-sacs, heavily infected with virus, were removed and ground with Pyrex fragments plus broth to 10% suspension. This was centrifuged 1 hour at 2000 RPM; the supernatant was recentrifuged in the cold for 2 hours at 12,000 RPM and the sediment obtained thereby was resuspended in saline to 10 times the volume of the original 10% yolk-sac suspension for use in the test. This was called "Lygranum" (Y.S.) antigen. The resuspended sediment from normal yolk-sacs, treated in the same way, was used as control.

The sera were inactivated at 56°C before use and dilutions were made in saline. The source of complement was pooled guinea-pig serum kept frozen at -32°C . Previous to each test the thawed complement was titrated and diluted in saline so that 2 hemolytic units were contained in 0.2 cc. In the test 0.2 cc of each reagent was added to the tubes in the following order: serum dilution, complement, and antigen. The well-shaken mixtures were placed for $1\frac{1}{4}$ hours at 37°C . Then to each tube was added 0.2 cc of 3% suspension of washed sheep cells sensitized with 2 minimal hemolytic doses of anti-sheep cell rabbit amboceptor. Readings for hemolysis were made after a further period of 30 minutes at 37°C . Controls for free complement and for anticomplementary action in each antigen and serum were always included. The titre of a given serum

² Shaffer, M. F., Rake, G., and McKee, C. M., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **44**, 408.

³ Rake, G., McKee, C. M., and Shaffer, M. F., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **43**, 332.

was taken as the highest dilution showing complete or nearly complete fixation with specific antigen and no fixation with the antigen control prepared from the corresponding normal tissues. The results with "Lygranum" (Y.S.) ran entirely parallel to those obtained with "Lygranum" (M.L.), although under the conditions of concentration employed in these experiments, the former antigen seemed more active. On the other hand, non-specific fixation in low serum dilutions with the antigen control from normal tissue was less frequently encountered in the case of mouse-lung. In performing the test the lowest initial serum dilution was 1:2 and since, in the system for fixation, this underwent a further threefold dilution, the lowest possible titre in these experiments was 1:6.

Using the method outlined, sera from 20 individuals with clinical history of lymphogranuloma venereum and positive reactions to Frei antigen were tested. Nineteen sera fixed complement specifically in the presence of "Lygranum" antigen, at titres ranging from 1:15 to 1:600. In one case the titer was only 1:6. The possible correlation between serum titre, degree of reactivity to standardized Frei antigen and the clinical status of lymphogranuloma infection in a group of cases is being investigated in collaboration with Dr. A. W. Grace of the New York Hospital, through whose courtesy most of the lymphogranuloma sera were obtained. As a control, sera taken from 22 presumably normal individuals were tested; 20 failed to fix complement even in the lowest dilution (titre less than 1:6). Positive reactions in this group were obtained only with serum from one laboratory worker who had been exposed to contact with the virus over the period of a year and with the serum of a female child residing in an orphanage.

As a further control, the test was carried out on a group of 29 sera with strongly positive Wassermann reaction obtained through the courtesy of Mr. J. V. Mulcahy of the State Dept. of Health, Trenton, N. J. and Dr. B. Webster of the New York Hospital. Sixteen of these sera gave specific fixation with "Lygranum" antigens showing titres between 1:15 and 1:150. In view of the reports concerning positive reactions to the Frei antigen, elicited in prostitutes who were tested as a matter of routine although not suffering overtly from lymphogranuloma, as well as the positive fixation by the serum of one worker in our laboratory and 55% of syphilitic sera which we have tested, the likelihood of undiagnosed or subclinical infection appears great and is being further investigated in collaboration with Dr. A. W. Grace.

If the reaction is specific, as we believe, it will prove to be a most

useful method for the detection of lymphogranuloma venereum infection particularly in individuals who have not been tested with Frei antigen, as well as for immunological studies in infections of humans and animals with this etiological agent.

Summary. In individuals with lymphogranuloma venereum, the serum has been found to fix complement regularly in the presence of antigens containing the virus in high concentration. Such fixation was observed only once with sera taken from supposedly uninfected individuals but was obtained frequently in syphilitic sera showing markedly positive Wassermann reactions.

11474

Persistence of St. Louis Encephalitis Virus in the Brains of Chicks.*

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St. Louis encephalitis virus has been cultivated *in vitro* on minced chick embryonic tissue¹ as well as in the yolk² and on the chorio-allantoic membrane^{1, 3-5} of chick embryos. On chorio-allantoic membranes, serial passage through 68³ and more than 100⁶ transfers has been possible. Brains of the corresponding embryos have been found to contain slightly more virus than the membranes, virus being also present in the livers and spleens.^{3, 5} Virus has been demonstrated in the brains of chicks allowed to hatch¹ though only slight or no microscopic changes were demonstrable.^{1, 3} It has been observed that embryos may survive until the time of hatching,³ or die within a few days after inoculation.^{1, 6}

Harrison and Moore¹ have reported that young chicks (4 to 6

* Studies supported by Mary Hooper Somers Fund for Filtrable Virus Research.

¹ Harrison, R. W., and Moore, E., *Am. J. Path.*, 1937, **13**, 361.

² Stimpert, F., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 483.

³ Smith, M. G., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 191.

⁴ Smith, M. G., and Lennette, E. H., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 323.

⁵ Schultz, E. W., Williams, G. F., and Hetherington, A., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 799.

⁶ Schultz, E. W., *et al.*, unpublished work.

days old) were found somewhat susceptible. After inoculation with mouse brain virus, 3 out of 6 showed clinical (paralysis) or microscopic evidence of infection, or both, and virus was recovered from the brains of 3 out of 4 of the chicks tested for virus.

The present report deals with the susceptibility of young chicks to

TABLE I.
Results Obtained on Inoculating Suspensions of Chick Brains into Mice.

Days after inoculation chicks were killed	Dilution of chick brain employed	Death of mice in days		
2	1:10	6	6	7
	1:100	6	6	6
2	1:10	6	6	*
	1:100	6	8	—
	1:1000	8	10	—
	1:10,000	—	—	—
4	1:10	5	7	*
	1:100	5	5	12
4	1:10	5	6	—
	1:100	6	—	—
	1:1000	—	—	—
6	1:10	4	7	11
	1:100	7	8	—
6	1:10	8	10	—
	1:100	6	8	8
	1:1000	—	—	—
8	1:10	6	—	—
	1:100	—	—	—
10	1:10	5	8	8
	1:100	8	—	—
10	1:10	6	—	—
	1:100	—	—	—
12	1:10	7	8	9
	1:100	11	16	*
	1:1000	—	—	—
12	1:10	—	—	—
17	1:10	10	—	—
20	1:10	8	10	—
21	1:10	—	—	—
31		—	—	—
42		—	—	—
56		—	—	—

Six survivors were discarded.

— Survived.

* Accidental death.

mouse brain passage virus. White leghorn, barred rock and Rhode Island red chicks were tested for susceptibility. The presence of virus in brains, livers or spleens of the chicks was determined by inoculating 3 mice each intracerebrally with 0.03 cc of a suspension (usually 10%) of material. Surviving test mice were observed for 21 days. All virus suspensions were also tested for bacterial sterility.

Webster's No. 3 strain of the virus was used. It regularly killed mice when inoculated intracerebrally in a dilution of 10^{-5} .

Of forty 2-day-old leghorn chicks which were inoculated subdurally with 0.03 cc of a 10% suspension of mouse brain virus, none showed any sign of infection. The object of the investigation then was to determine whether or not the brains harbored active virus. The chicks were killed 2 at a time at intervals of several days over a period of 56 days. The brains of the 2 chicks were pooled, ground in a mortar and 3 mice were inoculated with each dilution. In some cases one brain only was ground, while the other was sectioned for microscopic examination. The results of the tests for virus are given in Table 1.

Histological examination of the brains of 11 chicks, not tested for virus, but killed on the 2nd to 20th day after inoculation showed only slight, and inconstant changes, consisting at most of small, perivascular, mononuclear infiltrations. The livers, spleens, and kidneys of these animals in all cases appeared normal. No changes were observed in the brains and other tissues from chicks killed more than 20 days after inoculation.

Groups of 4 to 6 chicks, 2 days old, were then inoculated by various routes with 10% mouse brain virus. None of these showed any sign of infection. They were killed at intervals and the tissues pooled and tested for virus. No gross changes were noted in any of the tissues. Microscopic sections of brains were stained by Lentz A method, other tissues with hematoxylin and eosin. The observations on this group are given in Table II.

Ten leghorn chicks, 21 days old, were inoculated subdurally, each with 0.03 cc of 10% mouse brain virus. These showed no sign of infection over a period of one month.

Serial passage of virus was tried (using leghorns for the first 2 passages and barred rocks subsequently) in chicks 2 to 6 days old. Three chicks were inoculated subdurally each with 0.03 cc of 10% mouse brain virus. These were killed 3 to 4 days later. Their brains were ground together to make a 10% suspension and 0.03 cc were inoculated subdurally into each of 3 new chicks and intra-

TABLE II.
Results Obtained When Chicks Were Inoculated with a 10% Suspension of Mouse Brain Virus.

No. of chicks inoculated	5 Leghorns	4 Barred Rocks	5 Barred Rocks	6 R. I. Reds	6 R. I. Reds
Route and amount inoculated	0.3 cc subcutaneously	0.03 cc subdurally	0.3 cc intraperitoneally	0.03 cc subdurally	0.4 cc intraperitoneally
Days after inoculation chicks killed	30	28	28	21	21
Results on inoculating tissues into mice were negative.	3 brains	(a) 2 brains	3 brains	3 brains	3 brains
Kind of tissue (pooled)	3 (livers spleens)	3 (livers spleens)	3 (livers spleens)	(1/2 of each)	3 (livers spleens)
Microscopic examination of tissues of chicks which were not tested for virus	(b)	(b)	(c)	(d)	(e)

(a) One of 3 mice died

(b) No changes observed in brains, livers or spleens

(c) One brain had a small area of mononuclear cell infiltration in the choroid plexus; a second had a similar area but perivascular in distribution.

(d) Of 3 brains sectioned (in this case half of each brain was tested for virus), 2 showed several small foci of mononuclear cell infiltration, mostly in the cerebellum at the level of Purkinje cells.

(e) Brains not examined. Livers and spleens showed no changes.

TABLE III.

No. of the serial passage	1			2			3			4			5		
Death of individual mice in days	5	5	5	6	6	7	8	8	10	19	—	—	7	7	12
No. of the serial passage	6			7			8			9 10 days			9 25 days		
Death of individual mice in days	4	9	14	9	10	*	7	7	7	7	8	—	—	—	—
— Survival															
* Accidental death															

cerebrally into 3 mice. None of the chicks showed any sign of infection. Six instead of 3 chicks were used in the 9th passage; 3 of these were killed 10 days and three 25 days after inoculation. The results of the mouse inoculation with material from each serial chick passage are given in Table III.

Microscopic sections of the 3 brains of chicks from the 9th passage sacrificed 10 days after inoculation showed several foci of mononuclear cell infiltration near the meninges, some diffuse mononuclear cell infiltration and perivascular infiltration in various areas and particularly in the cerebellum.

Serial passage in chicks 2 to 6 days old was also attempted, in which the initial inoculation consisted of 0.5 cc of a 10% suspension of mouse-brain-virus administered intraperitoneally to each of 3 chicks. Transfers were then made by grinding all the brains and spleens together to make a 10% suspension and inoculating each of 3 new chicks with 0.5 cc intraperitoneally and 3 mice each with 0.03 cc intracerebrally. None of the chicks showed any sign of infection. Virus was recovered from the first passage, but not from the subsequent 8 passages.

Summary. Two-day-old chicks, after inoculation with St. Louis encephalitis virus, subdurally or by other routes, failed to show any clinical evidence of infection. Nevertheless, the brains of chicks which had been inoculated subdurally proved infectious for mice in a dilution of 10^{-2} for at least 6 days and sometimes in a dilution of 10^{-1} up to 20 days. Histological sections of such brains showed only slight changes, consisting at most of small areas of perivascular infiltrations.

The virus was carried through nine serial passages at 3- to 4-day intervals in the brains of 2- to 6-day-old chicks. Chick brains of the ninth serial passage showed areas of focal, diffuse and perivascular infiltration.

Effect of Breathing Pure Oxygen on Respiratory Volume in Humans.*

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Although previous investigators have concluded that the respiratory volume is not altered by the inspiration of high concentrations of oxygen^{1, 2} we have found an increase in the average respiratory volume in normal males when pure oxygen was breathed.

A group of 33 white male students, ages 18-33, served as subjects (mean age $23.5 \pm .8$). Each subject was given a preliminary trial on the day before the experiments were conducted and was then tested with oxygen at the corresponding hour of 2 subsequent days. In each experiment the subject rested for 20 minutes in the supine position and no tests were run sooner than one hour following a meal. Expired air was collected through a Siebe-Gorman half-mask and mercury valves (opened by a pressure of 1.5 mm of water), into a pair of spirometers of the Tissot type, each with a capacity of 9.19 liters (at 0°, 760 mm). The apparatus, which is described in detail elsewhere³ was arranged so that the time was electrically recorded when 9.19 liters of air were expired. Expired air was measured continuously over a period of 15 minutes before the administration of the pure oxygen and for 30 minutes during the inspiration of oxygen.

Oxygen was obtained in 1150-gallon pressure tanks from which a pair of Tissot spirometers, each with a capacity of 60 liters, was filled. In this way the oxygen was allowed to come to the same temperature and pressure as room air before being breathed. The valve system was arranged so that the change in inspired air from 21% to 100% oxygen could be made without the knowledge of the subject. Each experiment consisted of: (1) a 20-minute rest period in the supine position; (2) a 15-minute period for the measurement of respiratory volume with the subject breathing outdoor air; (3)

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¹ Benedict, F. G., and Higgins, H. L., *Am. J. Physiol.*, 1911, **28**, 1.

² Davies, H. W., Brow, G. R., and Binger, C. A. L., *J. Exp. Med.*, 1925, **41**, 37.

³ Shock, N. W., and Ogden, E. Child Development. In press.

a 20-minute period for the measurement of respiratory volume with the subject breathing 100% oxygen. In 36 experiments a fourth period of 15 minutes of air collection was continued with the subject again breathing outdoor air from the larger spirometers.

Respiratory volumes were computed in liters per minute at 0° C and 760 mm Hg. The resting level was determined for each experiment from the average of at least 8 observations. The change in respiration resulting from breathing pure oxygen was expressed as

TABLE I.
Effect of Breathing 100% O₂ on Resting Respiratory Volume

Resting Respiratory Volume.							
Subject No.	Test I.			Test II.			I. and II.
	Breathing	Breathing	% Incre- ment	Breathing	Breathing	% Incre- ment	Avg. % Incre- ment
	21% O ₂ 1./sq m/ min	100% O ₂ 1./sq m/ min		21% O ₂ 1./sq m/ min	100% O ₂ 1./sq m/ min		
7	2.95	3.14	6	2.87	2.75	4	1
8	3.80	4.11	8	4.02	4.13	3	6
9	4.16	4.05	- 3	5.41	5.88	9	3
10	2.97	3.04	2	3.32	3.73	13	8
11	3.75	4.35	16	4.38	5.14	17	17
12	3.65	4.11	12	3.31	3.64	10	11
13	4.12	4.32	5	3.85	3.98	3	4
14	4.16	4.39	6	3.64	4.10	13	10
15	3.08	3.45	12	2.95	3.28	11	12
16	3.26	4.02	23	3.33	3.95	18	21
17	3.56	4.85	36	3.45	4.17	21	29
18	2.55	2.62	3	2.41	2.51	4	4
19	3.10	4.26	37	3.48	4.46	28	33
20	4.67	3.73	-20	4.10	4.27	4	- 8
21	3.18	4.08	28	3.12	3.63	16	22
22	3.38	4.04	20	3.39	4.29	26	23
23	2.52	2.89	32	2.29	2.62	14	23
24	3.67	4.57	24	3.88	4.52	17	21
25	2.52	2.89	15	2.46	3.06	24	20
26	2.98	4.93	66	3.63	3.41	- 6	30
27	4.01	4.23	5	3.78	4.30	14	10
28	3.84	3.97	3	4.21	4.42	5	4
29	2.48	3.02	22	2.59	2.83	9	16
30	2.66	3.29	24	2.83	3.48	23	24
31	3.52	3.70	5	3.32	3.36	1	3
32	2.97	3.05	3	3.07	2.94	- 4	- 1
33	3.59	4.15	15	3.61	3.90	8	12
34	3.06	3.26	7	3.35	3.41	2	5
35	2.55	2.59	1	2.47	2.46	0	1
36	2.70	3.60	33	3.39	3.64	7	20
37	3.25	3.31	2	2.92	3.56	22	12
38	3.30	3.60	9	2.60	3.99	54	32
39	3.46	3.77	9	3.35	4.02	20	15
Mean	3.32	3.75	14.1	3.36	3.75	12.2	13.4
					S.D.	Mn.	1.8
						C.R.	7.5

a percentage deviation from the resting value obtained in the period prior to the breathing of oxygen. Since similar results were obtained when computations were based on the period of breathing air after the administration of oxygen, it is clear that the results cannot be attributed to increased restlessness of the subjects during a prolonged experiment.

Results are shown in Table I. It may be seen that in all but 5 experiments in the total of 66 an increase in respiratory volume occurred with the inspiration of pure oxygen. The average increment was 13.4% for 66 experiments with 33 subjects. Statistical tests indicate that this average increment would occur by chance only once in 1×10^{-6} trials.

The cause of this increase in respiration is speculative but the following possibilities are suggested: (1) because of the increased oxygen tension in the blood, less oxyhemoglobin is reduced in the tissues, thus releasing less base for CO_2 transport from the tissues. In this way the CO_2 tension of the respiratory center itself may be increased with a resulting increase in respiratory volume;⁴ (2) the increase in oxygen tension of the blood reduces cerebral blood flow⁵ which may result in a local increase in CO_2 in the respiratory center; (3) an increase in oxygen tension in the respiratory center may increase the sensitivity of the center to the normal stimulus so that respiratory volume is increased although no rise in $[\text{H}^+]$ or pCO_2 occurs.⁶

Summary. Breathing pure oxygen causes a significant rise in the average resting respiratory volume in normal males.

11476 P

Thermo-coagulation in Destruction of Tissue in Cerebral Cortex of Small Animals.

L. A. PENNINGTON (Introduced by J. F. Fulton)

From the Psychological Laboratory, University of Illinois.

In order to overcome certain mechanical difficulties inherent in the trephining method when applied particularly to the removal of tissue in the none too accessible auditory areas of the rat's cerebral hemispheres, the technic about to be described was devised.

⁴ Gesell, R., *Am. J. Physiol.*, 1923, **66**, 5.

⁵ Lennox, W. G., and Gibbs, E. L., *J. Clin. Invest.*, 1932, **11**, 1155.

⁶ Eastman, W. J., *International Clinics*, Series 46, 1936, **11**, 275.

Although Dennis and Bolton¹ suggested certain advantages of the thermo-coagulation method for the induction of lesions in the rat's brain, they made no attempt to describe in detail the procedures used to induce circumscribed lesions of varying magnitudes and depths in the brains of small animals frequently used in laboratories. The present technic, in brief, has involved the application of a heated platinum wire to a selected area of the exposed skull bone. This wire is left upon the skull for an interval, the magnitude of which depends upon the nature and purpose of the experiment. Microscopic study of the cerebral tissue, following sectioning and staining, indicates clearly that differential destruction of specific cortical layers may be effected by varying the duration of the application of the heated cautery wire to the external surface of the skull. Careful observation of other layers of the cerebrum indicates that the lesions so induced are clearcut and that adjacent cells are normal in appearance.

The data available have been accumulated from the study of the cerebral areas of 35 rats. These animals, all male albinos, were 3 months of age at the time that the cortical operations were performed. The results indicate that the application of the heated cautery tip to the skull for a period of 10 seconds effects the destruction of the outer or first cellular layer of the cortex within the auditory area.² If the wire is applied for 20 seconds all cortical layers within this region underlying the tip are destroyed. With an interval of 15 seconds the first five layers are destroyed. Twenty-five and 30-second intervals induce well delimited lesions which extend into the hippocampal regions. A detailed analysis of other areas and the intervals essential for the induction of cortical lesions of differing depths is in progress.

The cautery unit utilized for the induction of these brain lesions by thermo-coagulation has been devised for use in dentistry.* The heat obtained from this instrument is generated by alternating current. The unit is equipped with a dial which can be set for the regulation of the degree of heat generated by the electric current passing through the cautery tip acting as a resistance. A more accurate quantification of the power dissipated in the cautery tip has been made by means of the voltmeter-ammeter method. The calories of heat per second generated by the cautery tip have been determined for each dial reading.

¹ Dennis, W., and Bolton, C., *Science*, 1935, **81**, 297.

² Pennington, L. A., *J. Comp. Neurol.*, 1937, **66**, 415.

* Cautery unit devised and patented by Burton Manufacturing Company, Chicago.

Additional comments concerning the behavioral data obtained from this study are relevant at this point. These data obtained from carefully controlled observations of the animals in an auditory discrimination apparatus³ were in general agreement with those obtained from the observations of other animals operated upon by the extirpation method. Although these 2 sets of operative and behavioral data were not strictly comparable because of differential extents and depths of the lesions, it is clear that the method of thermo-coagulation results in postoperative disturbances in animal behavior similar in degree to those observed in animals of the other group.

That the values of this method are numerous seems clear. First, recovery of the animal is rapid. If the investigator wishes, the behavior of the animals may, in most instances, be studied from one to three days after operation. Rapid recovery makes possible a more detailed and complete collection of experimental data pertaining to the problem of the restitution of function following artificial injuries to the nervous system. Second, the procedure requires far less time for the actual performance of the operation, and, hence tends to reduce operative shock. This method does necessitate, however, the aid of an assistant who serves as a timekeeper. Third, the approach makes readily accessible, in the rat brain at least, certain cerebral areas, especially areas *j*, *k*, and *p* delimited earlier by Fortuyn.⁴ Fourth, the method results in fewer cases of infection and thus reduces the mortality rate. Fifth, the thermo-coagulation technic is more readily controlled than is the trephine method in studies on small animals. It makes possible the induction of very small or of very large cerebral lesions with fewer technical difficulties than is ordinarily possible with the extirpation method currently in use.

³ Hunter, W. A., and Pennington, L. A., *Science*, 1939, **89**, 87.

⁴ Fortuyn, A. B. D., *Arch. Neurol. and Psychiat.*, London, 1914, **6**, 221.

Respiration of Kidney Cortex in High Potassium-Low Sodium Ringer's Solution.*

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Carr and Beck¹ have shown that in the brief life span of an albino rat after bilateral adrenalectomy there is a gradual decrease of about 25% in the basal metabolic rate, when the animal is maintained on a "normal" diet. The experiments reported here are part of a series designed to determine the cause and effect relationship of this decreased metabolic rate in the syndrome of adrenal insufficiency. In this instance an attempt was made to see whether the typical serum electrolyte picture in advanced adrenal insufficiency would reduce the respiration of kidney slices from normal rats. The kidney was chosen for study because of its importance in producing the adrenal insufficiency syndrome² and because it has been shown by Crismon and Field³ that there is a decrease in kidney respiration in the adrenalectomized rat of about 38%.

Kidney slices were prepared with the Terry razor microtome which proved very satisfactory for this purpose.⁴ The oxygen consumption of kidney cortex was measured by the Warburg method.⁵ The suspension medium used in control experiments was the mammalian Ringer's of Dickens and Greville,⁶ hereinafter termed D.G.-Ringer's. For the experimental series the medium was a high potassium-low sodium modification of mammalian Ringer's solution osmotically balanced with glucose, hereinafter called A.I.-Ringer's. The concentrations of electrolytes in this medium were those reported for blood of adrenalectomized animals by Grollman⁷ and Hegnauer and Robinson.⁸ The compositions of these solutions are summarized in Table I.

* Supported in part by a grant from the Rockefeller Fluid Research Fund of the Stanford University School of Medicine.

¹ Carr, C. J., and Beck, F. F., *Am. J. Physiol.*, 1937, **119**, 589.

² Loeb, R. F., *Glandular Physiology and Therapy*, Chicago, The American Medical Association, 1935, Chapter 20.

³ Crismon, J. M., and Field, J., *Am. J. Physiol.*, 1940, in press.

⁴ Terry, B. T., *Am. J. Clin. Path.*, 1937, **7**, 69.

⁵ Field, J., Belding, H. S., and Martin, A. W., *J. Cell. Comp. Physiol.*, 1939, **14**, 143.

⁶ Dickens, F., and Greville, G. D., *Biochem. J.*, 1935, **29**, 1468.

⁷ Grollman, A., *The Adrenals*, Baltimore, Williams and Wilkins Co., 1936, 184-188.

⁸ Hegnauer, A. H., and Robinson, E. J., *J. Biol. Chem.*, 1936, **116**, 769.

TABLE I.

	D.G.-Ringer* g per liter	A.I.-Ringer* g per liter
NaCl	7.00	5.4
KCl	0.18	0.36
MgCl ₂ ·6H ₂ O	0.1627	0.1956
CaCl ₂ ·2H ₂ O	0.25	0.25
Glucose	2.00	9.80

*Both solutions were buffered at pH 7.4 with sodium phosphate in final concentration of M/150.

Thirty-two determinations were made in D.G.-Ringer's solution and 39 in the A.I.-Ringer's solution. Since the tissues were obtained from 9 white rats (Slonaker-Wistar strain), the arithmetic means of the results obtained on each animal were analyzed statistically as paired data. The mean oxygen consumption, N.P.T., per mg (dry weight) in one hour was 16.954 cu mm in D.G.-Ringer's solution and 16.098 cu mm in A.I.-Ringer's solution. The mean difference was 0.855; the standard deviation was 1.29; the standard error was 0.489, and the value of "t" (Fisher, 1936) was 1.7485. This indicates that this small difference in oxygen consumption could occur by chance more than 5 times in a hundred. Thus, the difference observed is not statistically significant. Although the mean difference observed may actually exist, as might possibly be shown by a more lenient statistical method, this observed fall in oxygen consumption, even at its maximum, is not of the order of magnitude of that observed for tissues of adrenalectomized animals.

It can readily be seen that this experiment represents a very limited reproduction of the situation occurring in adrenal insufficiency. Although the electrolyte content of the A.I.-Ringer's solution approximates the blood picture occurring in fairly extreme cases of adrenal insufficiency, there are certain aspects of the total picture which have necessarily been omitted. Thus, for example, the time factor has been completely neglected. Whereas the electrolyte change occurring in adrenal insufficiency is slow and chronic, this experiment, as a first approach to the evaluation of the electrolytic factors, necessarily represents an acute situation.

To the extent that the experimental situation produced here *in vitro* is comparable to the electrolytic imbalance occurring in adrenal insufficiency it would appear that the changed electrolyte content does not have a direct rôle of significant magnitude in the depression of the oxygen consumption of the kidney cortex.

Influence of Rate of Urine Formation on Potassium Excretion.*

V. E. HALL AND L. L. LANGLEY

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From an accurate description of the relations existing between the rate of renal excretion of any substance and the rate of urine formation, it is possible to make certain deductions concerning the manner in which the kidney excretes that substance. In spite of the active current interest in potassium metabolism, this relation as it exists in man has not been satisfactorily studied. We have attempted to fill this gap.

Repeated simultaneous determinations of plasma and urinary potassium concentrations were made upon 3 normal adult male subjects maintained on diets of approximately constant potassium content. The water content was varied within wide limits. With extreme water deprivation, urine flow rates as low as 0.5 cc per min. were obtained; while with ingestion of excess water, the flow reached 6 or more cc per min. Urine was collected for one 2-hour period, in the middle of which blood was drawn for analysis. The blood was oxalated and centrifuged immediately at 3000 rpm for 20 minutes. Potassium was determined on ashed urine and plasma by the method of Kramer and Tisdall.^{1, 2} Special precautions were taken to prevent loss of precipitate in washing. The subjects carried on regular laboratory work during the experimental periods.

The excretion rates have been expressed as clearances (C), calculated by the conventional formula:

$$C = \frac{U \cdot V}{P}$$

in which U is the concentration of potassium in the urine (mg per cc), V the volume of urine (cc per min.), and P the plasma potassium concentration (mg per cc). The plasma potassium concentrations (means with standard errors of means) were: Subject L, 16.4 ± 0.2 ; Subject C, 16.5 ± 0.6 ; and Subject H, 17.9 ± 0.4 mg per 100 cc.

From Fig. 1, in which the clearances are plotted against the rates of urine flow, it may be seen that, over a wide range of flow rates,

* Supported in part by a grant from the Fluid Research Fund of the Stanford University School of Medicine.

¹ Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, **46**, 339.

² Tisdall, F. F., and Kramer, B., *J. Biol. Chem.*, 1921, **48**, 1.

the clearance remains constant. Below about 0.6 cc per sq m per min ("the augmentation limit"), the clearance falls sharply in all 3 subjects. Since, according to Chesley,³ the glomerular filtration rate begins to fall off as the urine flow reaches approximately this value, it is probable that the decrease in potassium clearance at urine flow rates below the observed augmentation limit is due to a reduction in glomerular filtration.

The observations of Griffon⁴ apparently showed that the rate of potassium excretion in man was proportional to the rate of urine flow. However, he confined his observations to the range of 0.35 to 1 cc per min., and was thus working largely below the augmentation limit. On the other hand, the data of Cutler, Power and Kendall⁵ suggested that in normal human subjects potassium excre-

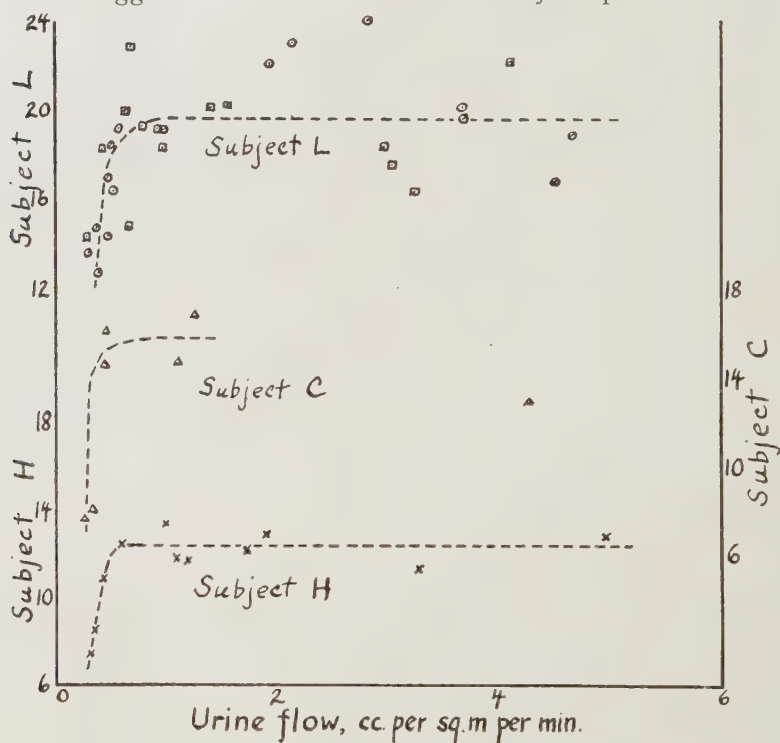


FIG. 1.

Effect of urine flow rate on potassium clearance in normal male subjects. Clearances (ordinates, with separate scales for each subject) are stated as cc plasma cleared per min.

³ Chesley, L. C., *J. Clin. Invest.*, 1938, **17**, 591.

⁴ Griffon, H., *Comp. rend. Soc. de Biol.*, 1936, **121**, 47.

⁵ Cutler, H. H., Power, M. H., and Wilder, R. M., *J. Am. Med. Assn.*, 1938, **111**, 117.

tion was independent of urine flow. All the flow rates of these investigators now appear to have been above the augmentation limit. Our observations confirm and reconcile the findings of both these groups of workers.

The clearance ("maximal") of our 3 subjects averaged about 16 cc per sq m per min. Since the glomerular filtration rate of normal subjects is about 69 cc per sq m per min, potassium must be reabsorbed by the kidney tubules. Since both the rate of glomerular filtration and the rate of potassium excretion remain constant over a wide range of urine flow rates, the rate of tubular reabsorption must also remain constant. Accordingly, the concentration of potassium in the tubular urine may vary widely without causing changes in the rate of tubular reabsorption. Such reabsorption cannot therefore be entirely a passive process resulting from the gradient established by the reabsorption of water.

The factors controlling such reabsorption are now under investigation in this laboratory.

11479 P

Hemolytic Action of Fluorides on Certain Nucleated Erythrocytes.

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In a study of CO₂ dissociation curves of dogfish blood (Ferguson, Horvath and Pappenheimer¹ it was observed that sodium fluoride added to prevent glycolysis caused a slowly progressive swelling, usually ending after 3 to 10 hours in hemolysis. Hemolysis of fish blood by oxalate has been observed by Black and Irving.² The effects of several fluorides, oxalates and other salts have been tested on the blood of various species with nucleated erythrocytes. One tenth molar and molar solutions of the salts were added to 9 times their volume of the blood to be tested, which had previously been defibrinated. The final concentration of the salts was, in one series, one-hundredth molar and, in the other, one-tenth molar. In the first series the final solution bathing the cells would be slightly

¹ Ferguson, J. K. W., Horvath, S. M., and Pappenheimer, J. R., *Biol. Bull.*, 1938, **75**, 381.

² Black, E. C., and Irving, Laurence, *J. Cell. Comp. Physiol.*, 1938, **12**, 255.

hypotonic and in the second slightly hypertonic. In the later experiments using molar solutions the more alkaline ones (oxalates, citrates and arsenates) were adjusted by the addition of HCl to a pH between 7.0 and 7.4, as indicated by phenol red. This procedure produced in certain cases significant alterations in the results. The following salts were tested on all the species used—sodium chloride, sodium thiocyanate, sodium fluoride, sodium oxalate. In some experiments the following salts were used, too—sodium citrate, sodium arsenate (mostly dibasic), aluminium fluoride, zinc fluoride. The bloods tested included dogfish (*Mustelus canis*), tautog (*Tautoga onitis*), sea robin (*Prionotus carolinus*), squeteague (*Cynoscion regale*) and a turtle and a snake (species unknown).

Results. Progressive swelling was not observed after the addition of sodium chloride or sodium thiocyanate. All the fluorides tested produced progressive swelling and (when the observations were sufficiently prolonged) hemolysis of the erythrocytes of all the fish listed above. The nucleated erythrocytes of the turtle and snake were not, however, susceptible to fluoride. The results with oxalate, citrate and arsenate were less regular. Sometimes swelling and hemolysis were produced and it occurred more often with the stronger concentrations. In a few experiments it appeared that even the stronger solutions failed to cause swelling when they were neutralized. This phenomenon suggested a possible explanation of the results.

Fluorides, oxalates, arsenates and citrates might be expected to remove magnesium and calcium ions from solution. The latter 3 anions would remove magnesium less effectively in neutral or acid solution, although they would still be effective in removing calcium ion. The removal of magnesium ion from the susceptible bloods might alter the permeability of erythrocytes in some manner which would result in the swelling and hemolysis *e.g.* by increasing the permeability to cations. However, these results are presented in this incomplete form not so much to indicate an explanation as to draw attention to a phenomenon which has complicated investigations of the transport of respiratory gases by fish blood. Circumstances make it unlikely that we shall be able to pursue this investigation further in the near future.

Summary. The nucleated erythrocytes of certain fishes show progressive swelling and eventual hemolysis on the addition of fluorides. Oxalates and arsenates produce a similar effect but with less regularity. The nucleated erythrocytes of a turtle and a snake did not react in this way.

Effect of Arsenicals on Liver Lipids of Rabbits.

P. L. MACLACHLAN. (Introduced by E. J. Van Liere.)

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Attempts to determine the effect of liver injury on the amount and distribution of the liver lipids have yielded widely divergent results. Theis¹ found that the relation of phospholipid to neutral fat is quite constant for normal liver tissue and may be expressed as an equilibrium, 55 to 60% phospholipid: 45 to 40% neutral fat. However, if the liver is damaged or diseased this relation is altered. The abnormal organs seldom show any change from normal in the amount of total lipid, but the proportion of phospholipid is greatly diminished apparently because of a failure to convert neutral fat to phospholipid. Results obtained by MacLachlan² for white rats are at variance with those reported by Theis in two respects: (1) the proportion of total lipid present as phospholipid in normal liver tissue is considerably higher, and (2) no displacement of the normal phospholipid: neutral fat balance takes place as a result of liver injury. MacLachlan and Hodge³ found in cocaine-fed mice which showed extensive liver injury that the neutral fat and cholesterol contents increase greatly but the phospholipid content remains strikingly constant. This clearly shows that a change in the phospholipid to neutral fat ratio of the liver lipids from normal may result from a change in the neutral fat content only.

Since arsenicals are capable of producing extensive necrosis of the liver with fatty degeneration, it was considered worthwhile to determine the effect of arsphenamine and neoarsphenamine poisoning on the amount and distribution of the liver lipids.

Fourteen young adult rabbits of both sexes were maintained on a diet of Purina rabbit chow for 2 weeks prior to the experiment. To each of 4 rabbits, 50 mg per kg of arsphenamine were administered intravenously every third day until 5 doses were given; to each of another 2 animals, 5 doses of 75 mg per kg of neoarsphenamine were administered similarly. Thus the rabbits received a total of 250 mg per kg of arsphenamine or 375 mg per kg of neoarsphenamine within 2 weeks. The remaining 8 animals served as controls.

¹ Theis, E. R., *J. Biol. Chem.*, 1928, **76**, 107; 1928, **77**, 75; 1929, **82**, 327.

² MacLachlan, P. L., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 31.

³ MacLachlan, P. L., and Hodge, H. C., *J. Biol. Chem.*, 1939, **127**, 721.

A small portion of each liver was used for moisture determination. Lipid analyses were made on another portion by standard procedures, Bloor⁴ and Boyd.⁵

Histological examination of the livers showed moderate to severe necrosis with fatty degeneration as a result of the arsenical treatment. However, the results of the chemical analyses of the livers (Table I) show that there are no significant changes from normal following the administration of either arsphenamine or neoarsphenamine. The neutral fat content of the treated animals, while showing more individual variation, is no greater in amount than that of the untreated animals. The constancy of the phospholipid content, moreover, does not support the idea that in liver injury neutral fat increases at the expense of phospholipid. The normal values obtained for the total lipid content and the ratio of phospholipid to neutral fat of the livers of rabbits following arsenical poisoning are in agreement with the observations of MacLachlan² for rats following liver injury, but stand in marked contrast to the findings of MacLachlan and Hodge³ for cocaine-fed mice. Apparently a change in the phospholipid : neutral fat balance of the liver

TABLE I.
Liver Lipids of Rabbits Following Administration of Arsphenamine and Neoarsphenamine. (Calculated on the basis of moist weight.)

Rabbit No.*	Moisture, %	Total Lipid, %	Phospholipid, %	Neutral Fat, %	Cholesterol, %	Phospholipid : Neutral Fat %
1-C	71.9	4.42	3.15	.928	.337	71 : 21
2-C	71.1	4.68	3.43	.873	.381	73 : 19
3-C	71.7	4.68	3.68	.666	.331	79 : 14
4-C	72.0	4.44	3.44	.669	.335	78 : 15
5-C	73.3	3.95	3.07	.462	.415	78 : 12
6-C	70.7	4.23	3.45	.487	.297	82 : 12
7-C	71.5	4.01	3.22	.516	.278	80 : 13
8-C	70.4	4.15	3.23	.569	.350	78 : 14
Avg	71.6	4.32	3.33	.646	.341	77 : 15
9-A	73.7	4.12	3.16	.361	.600	77 : 9
10-A	74.3	4.11	3.44	.237	.433	84 : 6
11-A	72.0	4.43	3.62	.372	.437	82 : 8
12-A	70.5	3.74	2.47	.995	.277	66 : 26
13-N	72.0	4.74	3.29	.960	.390	70 : 20
14-N	72.5	4.04	3.03	.645	.365	75 : 16
Avg	72.5	4.20	3.17	.595	.417	76 : 14

*C—Control; A—Arsphenamine; N—Neoarsphenamine.

†Expressed as per cent of total lipid.

⁴ Bloor, W. R., *J. Biol. Chem.*, 1928, **77**, 53.

⁵ Boyd, E. M., *J. Biol. Chem.*, 1931, **91**, 1.

following liver injury occurs only when there is a change (from normal) in the total lipid content of the organ.

Summary. Liver injury in rabbits resulting from the administration of arsenicals in the form of arsphenamine and neoarsphenamine cause no significant changes from normal in the amount or distribution of the liver lipids.

The author wishes to express his appreciation to Dr. G. A. Emerson of the Department of Pharmacology for furnishing the experimental material and to Dr. J. E. Andes of the Department of Pathology for the histological examination of the tissues.

11481

Effect of Chlorination of City Water on Virus of Poliomyelitis.*

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Water was considered in early reports concerning the transmission of poliomyelitis. This method of spread seemed unlikely when later experimental evidence favored an air-borne infection entering the host through the olfactory tract. However, Kling,¹ observing European epidemics, reconsidered the question and additional evidence was accumulated incriminating water as a factor in the spread of the virus.

Poliomyelitis virus was found in human feces as early as 1912² and these observations have been amply confirmed. Unfortunately the technic of Sawyer³ requiring a second monkey passage as an important criterion to verify the presence of the virus was ignored until 1938. In that year, Trask, Vignec, and Paul,⁴ and Kramer, Hoskwith, and Grossman⁵ improved the technic of virus isola-

* This work was aided by a grant from the Clara Ward Seabury Clinic for the Study of Infantile Paralysis.

We are indebted to Mr. Harry McEntee, Supervising Chemist, Ann Arbor Water Softening Plant, for valuable assistance in this work.

¹ Kling, C., *Bull. Office internat. d'hyg. pub.*, 1928, **20**, 1779.

² Kling, C., Petterson, A., and Wernstedt, W., *Communication Inst. méd. État*, Stockholm, 1912, **3**, 5.

³ Sawyer, W. A., *Am. J. Trop. Dis. and Prev. Med.*, 1915, **3**, 164.

⁴ Trask, J. D., Vignec, A. J., and Paul, J. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, **38**, 147.

⁵ Kramer, S. D., Hoskwith, B., and Grossman, L. H., *J. Exp. Med.*, 1939, **69**, 49.

tion and included serial passage in monkeys. Using the new procedure Paul, Trask, and Gard⁶ detected the virus in sewage in the Charleston and Detroit epidemics of 1939. In addition, Kramer, Gilliam and Molner⁷ isolated the virus from stools of healthy contacts in a Detroit institutional outbreak. Others⁸⁻¹² during the past year have succeeded in isolating the virus and carrying it through a second animal passage.

The presence of the virus in human intestinal discharges led Levaditi, Kling and Lépine¹³ to investigate the effect of chlorination. A concentration of 4 parts per million (ppm) destroyed the virus in a cloudy tap water emulsion of infected monkey cord in 24 hours; 0.40 ppm was equally effective with clarified preparations. The pH and temperature of the emulsions were not recorded. They concluded that chlorination by the usual methods was virucidal. This work is lacking in two essentials, namely, the minimum effective chlorine concentration, and the shortest effective contact period. The chlorine concentrations and contact periods they used were considerably in excess of those employed in this country. Because of lack of data on the virus-inactivating effects of chlorination as usually practiced, the problem was reinvestigated.

Fresh water was obtained for each experiment from the Ann Arbor Water Softening Plant where the water is treated by the ammonia-chlorine process in which chlorine is present as chloramines. The chlorine content of the water was determined by the ortho-tolidine test. Reducing substances in the water did not interfere with its accuracy. The MV virus was selected for these experiments. It had a minimal infective dose of approximately 0.001 g of spinal cord.

The suspensions to be tested were prepared by making a 10% emulsion of infected spinal cord in saline with subsequent centrifugation at 4,500 rpm (radius 10 cm). The supernatant contained a minimum amount of organic matter. This was desirable since

⁶ Paul, J. R., Trask, J. D., and Gard, S., *J. Bact.*, 1940, **39**, 63.

⁷ Kramer, S. D., Gilliam, A. G., and Molner, J. G., *Public Health Rep.*, 1939, **54**, 1914.

⁸ Lépine, P., and Sédallian, P., *Comp. rend.*, 1939, **208**, 129.

⁹ Toomey, J. A., *Arch. Ped.*, 1939, **56**, 693.

¹⁰ Howe, H. A., and Bodian, D., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 538.

¹¹ Stimpert, F. D., personal communication.

¹² Kempf, J. E., and Soule, M. H., unpublished.

¹³ Levaditi, C., Kling, C., and Lépine, P., *Bull. Acad. de méd.*, Paris, 1931, **105**, 190.

Mallman¹⁴ and Fox¹⁵ demonstrated a protective effect on bacteria of organic matter in the presence of chlorine.

In experiment 1, 2.0 cc of the supernatant were added to 100.0 cc of chlorinated water and the same amount to a distilled water control. The initial chlorine content at the time of adding the virus was 0.58 ppm; this dropped to 0.10 ppm in an hour at which time 2.0 cc of each solution were injected intracranially into monkeys. Both animals developed quadriplegia, the control in 6, the other in 8 days.

Experiments 2 and 3 (Tables I and II): One part of the virus suspension was diluted with 165 parts of chlorinated water; a distilled water control was also prepared. Five minutes later the chlorine content, temperature and pH were determined. At stated intervals, monkeys were inoculated as in Experiment 1. The temperature readings were made at the time of each monkey injection.

Attention should be directed to the persistence of a chlorine

TABLE I.
Effect of Chlorination on MV Virus in 1:1650 Dilution.†

Monkey No.	Chlorine concentration, ppm			Contact period, hr	Neurological signs	Time of onset, days
	Original Conc.	5 min after exposure to virus	At time of inoculation			
3	(Control)				Quadriplegia*	12
4	0.55	0.55	0.50	1½	"	35
5	0.55	0.55	0.35	4	Negative	
6	0.55	0.55	0.20	10	"	
7	0.55	0.55	0.05	24	"	

*Histopathological picture was compatible with that of acute poliomyelitis.

†Temperature 21-24°C; pH 8.5.

TABLE II.
Effect of Chlorination on MV Virus in 1:1650 Dilution.†

Monkey No.	Chlorine concentration, ppm			Contact period, hr	Neurological signs	Time of onset, days
	Original Conc.	5 min after exposure to virus	At time of inoculation			
8	(Control)				Quadriplegia*	10
9	0.80	0.55	0.40	1	"	14
10	0.80	0.55	0.40	2½	XI nerve paralysis*	
					Leg paralysis	10
					Arm paresis	
11	0.80	0.55	0.25	5	Negative	

*Histopathology was compatible with that of acute poliomyelitis.

†Temperature 21-23° C; pH 8.3.

¹⁴ Mallman, W. L., *Mich. Eng. Exp. Sta., Bull. No. 59*, 1934.

¹⁵ Fox, L. A., *Military Surgeon*, 1936, **78**, 329.

content of 0.50 ppm for 1½ hours in Experiment 2, indicating a negligible chlorine demand by the organic matter. In Experiment 3, apparently there was more organic material present because the residual chlorine dropped from 0.80 ppm to 0.50 in 5 minutes and to 0.40 ppm in 1 hour. The contact period required for inactivation of the virus was approximately the same in both instances. As an additional control 0.45 ppm was adequate to kill *B. coli* in a concentration of 24,000 organisms per cc in ½ hour.

In municipal practice, a residual chlorine content of 0.10 to 0.20 ppm for ½ to 2 hours is considered adequate for the production of a safe water. The results in this paper indicate that a higher concentration and a longer contact period are necessary to inactivate the virus of poliomyelitis. The possibility that drinking water, adequately chlorinated according to accepted standards, may be a factor in the epidemiology of poliomyelitis must be recognized as a result of these findings. As a corollary, attention is directed to the shortcoming of this method for the protection of swimming pool water since carriers may discharge the virus from the intestinal tract or the naso-pharynx and the chlorine content of swimming pools is apt to drop significantly during the peak bathing loads. The need for more sensitive methods for detecting the poliomyelitis virus in water should be emphasized. Even persistently negative results would not necessarily assure the absence of the virus from water, because organisms such as *B. typhosus* are seldom found by direct bacteriological methods.

Whether the aluminum hydroxide sedimentation process previous to chlorination would produce virus-free water cannot be answered in this paper. Experiments are being continued to determine whether the chlorine concentrations usually used in swimming pools are sufficient to inactivate the virus.

Summary. Chlorine in a concentration of 0.5 ppm, which is an amount in excess of that usually employed in municipal practice, did not inactivate the virus of poliomyelitis in 1½ hours.

Non-Induced Cardiopathic Disease in a Rabbit—Electrocardiographic and Pathologic Study.

JAN NYBOER. (Introduced by Maurice Bruger.)

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The recognition of heart disease in experimental animals would be a great asset in the selection of healthy animals. Miller¹ has shown that spontaneous interstitial myocarditis existed in rabbits. Reference to the electrocardiographic diagnosis of non-induced cardiac disease in laboratory animals was not found in the literature. However, many electrocardiographic studies on induced heart disease have been made.²⁻⁴ Seifried⁵ referred to pathologic studies in rabbits with heart disease.

In a preliminary control electrocardiographic study on 16 three-month-old rabbits, there was a definite variation in the voltage, rhythm and form of the electrical complexes. The electrocardiogram of rabbit No. 7 in this series diverged definitely from the average so that a diagnosis of acute myocardial disease was suggested. This tracing also indicated the possibility that the disease might be localized in the myocardium.

This rabbit was received in a shipment one week previously and no known experiments had been performed on it. General observations showed the animal to be drowsy, inactive, and anorexic. The rectal temperature was 103° F. on the day the electrocardiogram was taken. Inanition continued for 2 days. On the third, the rabbit was found dead in its cage. On autopsy, a gross inspection of the body, lungs and abdominal viscera showed no demonstrable pathology. The epicardium, however, was adherent to the right anterolateral chest wall. No pericardial effusion was present. By comparison with normal hearts it measured about the same size. The heart was placed in formalin, sectioned and stained with hemotoxylin and eosin for microscopic study.

Figure I. shows electrocardiograms of a control rabbit and of

¹ Miller, C. P., *J. Exp. Med.*, 1924, **40**, 524.

² Agduhr, E., and Stenstrom, N., *The Appearance of the Electrocardiogram in Heart Lesions Produced by Cod Liver Oil Treatment*, Almquist and Wiksells, Uppsala, 1930.

³ Johnston, F. D., Hill, I. G. W., and Wilson, F. N., *Am. Heart J.*, 1935, **10**, 903.

⁴ Wood, F. C., and Wolferth, C. C., *Arch. Int. Med.*, 1933, **51**, 771.

⁵ Seifried, O., *Krankheiten des Kannichens*, Julius Springer, Berlin, 1937.

rabbit No. 7. The control animal exhibited only slight variations in the contours of its electrocardiograms during 4 months of observation. Significant variations in the standard leads (I, II, III) and of the exploring right and left chest leads paired with the indifferent left leg electrode were observed in rabbit No. 7 as compared with the control observations.

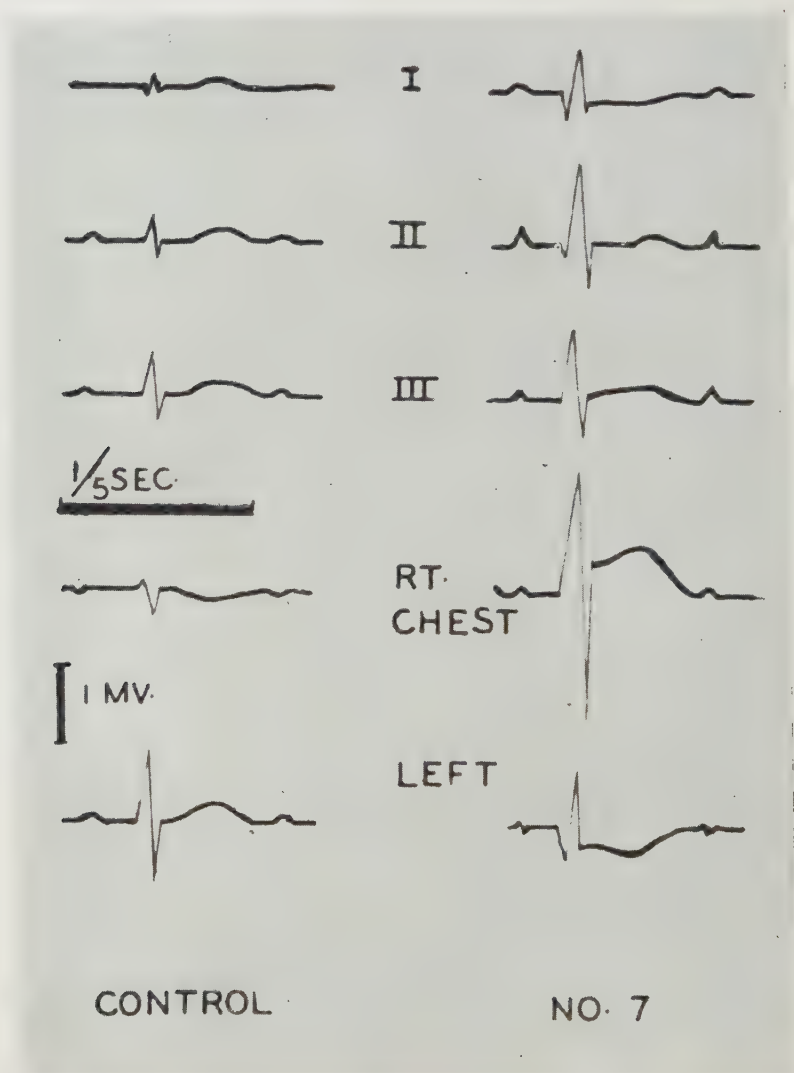


FIG. 1.

Diagram of a normal (control) and the abnormal rabbit electrocardiograms (rabbit No. 7) taken in all leads at normal sensitivity and adapted to the new terminology for the exploratory leads.



PLATE 1.

A section through rabbit No. 7 myocardium, approximately midway between the apex and the auricular junction. Low power magnification.

The chief objective differences between these graphs were defined by describing the deviations in the abnormal electrocardiogram of rabbit No. 7. The presence of the Q_1 of 2.8 mm, the depressed RS- T_1 of 1.7 mm, the inverted T_1 of 1.7 mm, the elevated RS- T_3 of 1.4 mm were probable deviations from the normals, but the T_2 and T_3 in the standard leads were upright and not definitely

abnormal. The right chest exploring lead showed no evidence of a Q wave, but a markedly elevated RS-T segment, associated with an upright T wave. The left chest exploring lead showed a deep Q wave and a markedly depressed RS-T segment associated with an inverted T wave. These RS-T deviations are definitely abnormal. The voltages of the chief QRS deflections in all the leads appeared greater than those observed in normal rabbits. In general, the rate of 300 per minute was faster than the average of normal rabbits studied.



PLATE 2.

Magnification of Plate 1 section in the marked inset showing scattered areas of normal, necrobiotic, and necrotic tissue beneath the endocardium and of the papillary muscle.

Microscopic sections through different levels of the myocardium of a normal rabbit showed the muscle bundles sharply outlined and the nuclei well stained. No evidence of degeneration or inflammation was present. Sections through the myocardium of rabbit No. 7 showed (Plates 1 and 2) areas of necrosis everywhere, chiefly affecting the muscle and largely involving the right ventricle and interventricular septum. In the left ventricle the areas of necrosis were chiefly beneath the endocardium. The papillary muscles were prominently affected. Slight periarterial infiltration was present and areas of necrosis were seen in both the auricular walls. The anterolateral ventricular epicardium showed a hyaline fat necrosis. The pathological diagnosis was degeneration and necrosis of the myocardium.

Summary. The incidence of non-induced cardiopathic disease among laboratory animals may greatly alter the prognosis, course and reaction to given control or experimental conditions. Disease of the myocardium as confirmed by pathologic studies may greatly alter the electrocardiogram in the rabbit. The changes found suggesting a localized lesion of the ventricle by the electrocardiogram were not supported by pathologic studies. Since bacteriologic studies were not done, no conclusive evidence as to the etiology of the myocarditis was suggested.

11483

Variability of Action on Heart Rate Compared with Metabolic Effect of Various Thyroid Preparations.

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In our previous publication¹ we have shown that certain thyroid preparations fed to thyroidectomized rats exert a stimulating action on the heart rate which varied from one product to the other in its relation to the corresponding metabolic increase obtained. While in 2 U.S.P. thyroid preparations the cardiac effect prevailed, thyroxine and thyroid globulin proved to be of low action on the heart if given at a dose to produce an equal metabolic response. It was shown furthermore, that alkaline hydrolysis of thyroid globulin

¹ Meyer, A. E., and Yost, M., *Endocrinology*, 1939, **24**, 806.

caused the formation of 2 split products, representing essentially the thyroxine and diiodotyrosine fractions, which differed widely in the degree of their metabolic potency, but which both showed a quite conspicuous action on the heart. It was concluded that hydrolysis produced a heart stimulator from either the thyroid hormone itself or from some unspecific substance contained in the material subjected to hydrolyzing agents. The question whether or not the heart stimulation obtainable with U.S.P. thyroid was caused by a substance contained originally in the gland, or to some split product formed by post-mortem changes could not be decided upon.

In continuation of this work we compared a number of other thyroid preparations, either dried gland powders of different commercial provenience or extracts prepared from thyroid, with respect to metabolic effect and action on heart rate, using the same technic as described before,² which consisted in feeding the medication (calculated in gamma per 10 g body weight) for 3 days and determining the metabolic and heart effect on the fifth day.

Samples of dried whole thyroid (U.S.P.) used in our previous work gave the standard metabolic response of 30% increase at a dosage of 310 to 320 γ , corresponding to 0.62 to 0.64 γ of iodine. In the following experiments the dosage producing 30% metabolic

TABLE I.

Product tested	Actual iodine content of undiluted product, %	Quantity of material after dilution to 0.2% I content, producing 27-32% metabolic stimulation, γ	Quantity of thyroxine contained in quantity given in column 3 γ	Avg heart stimulation obtained at about 30% inc. of metabolism,		
				Before	After	Incr.
d,l-thyroxine as Na salt	65.0	244	.75	190 - 220	=	30
Thyroid Globulin						
No. 123	.565	260	.23	180 - 210	=	30
134	.78	293	.25	190 - 226	=	36
136	.72	310	.22	188 - 236	=	48
141	.44	275	.27	190 - 227	=	37
143	.76	289	.26	190 - 212	=	22
U.S.P. A.	.2	320	.30	185 - 340	=	155
W-1	.465	309	.25	190 - 365	=	175
U.S.P. W-2	.2	240	.19	192 - 310	=	118
W-3	.63	236	.20	195 - 284	=	89
U.S.P. C.	.23	320	.26	192 - 275	=	83
U.S.P. L.	.2	320	—	190 - 273	=	83
Thyroid Ext. P.	.3	300	—	190 - 275	=	85
Commercial Thyroid Protein	.94	446	.3	195 - 260	=	65

² Meyer, A. E., and Wertz, A., *Endocrinology*, 1939, **24**, 683.

response was determined in every instance and the effect on the heart rate obtained simultaneously was noted.

Since not all these preparations were U.S.P. and some had a higher iodine content, the products were diluted with milk-sugar to contain 0.2% iodine in order to obtain comparable figures.

The table giving the averages, obtained on 6-12 rats in each case, shows that the metabolic effect in a large percentage of preparations is proportionate to the iodine content, the standard dose being about 300 γ , but that deviations even in U.S.P. thyroid do occur, as shown in sample W-2 and W-3. The effect on the heart was not in proportion with the metabolic efficiency, confirming our previous findings that both effects are to some extent independent.

The commercial thyroid protein, claimed to be "detoxified," gave a relatively low heart stimulation but a 50% higher dosage was required for the standard metabolic effect. The thyroxine content of the products was determined by the Leland-Foster method.³ From the data presented in the table the ratio between thyroxine and metabolic effect seems to be slightly more variable than that between iodine and that action, the quantity in the dosage varying from 0.19 to 0.3 γ .

Incidentally, iodized protein and peptone supplied by Dr. W. T. Salter, Thorndike Memorial Laboratory, Boston, gave proportionate metabolic responses in agreement with clinical tests but induced practically no heart acceleration.^{4, 5}

The observation mentioned above that hydrolysis of the thyroid globulin produces split products of strong action on the heart was met with the criticism that this effect might be due to some unspecific product of decomposition obtainable by hydrolysis from any animal tissue and perhaps present in the commercial product of high effect on the heart as a consequence of autolytic changes in the structural elements of the gland tissue occurring before the drying process was completed.

To answer the question beef muscle was minced and subjected to hydrolysis. The water-insoluble part was extracted with alcohol and both aqueous and alcoholic extract combined and evaporated. The extract did not show any sign of metabolic effect when tested on rats nor did it affect the heart rate. It was admixed to a standardized thyroid globulin that at 85 γ dosage per 10 g given for 3 days produced a metabolic increment of 30% and heart rate increase of about 25 beats per minute. The figures obtained by the

³ Leland, J. P., and Foster, G. L., *J. Biol. Chem.*, 1932, **95**, 165.

⁴ Lerman, J., and Salter, W. T., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, **38**, 94.

⁵ Salter, W. T., and Lerman, J., *Trans. Assn. Am. Phys.*, 1938, **53**, 202.

use of the mixture were absolutely identical. The conclusion, therefore, is justified that the heart stimulator is not a split product obtainable by hydrolysis from this type of animal tissue.

Conclusions. The iodine content in thyroid preparations seems to be an approximate guide for the estimation of metabolic effect; however relatively large deviations do occur in some products. The effect on the heart is not related to the metabolic action. The thyroxine content has still less demonstrable proportionality to either physiologic effect. While hydrolysis of thyroid globulin increases its heart action hydrolysate from muscle tissue is inert in that respect.

11484 P

Growth-Stimulating Effect of Testosterone Propionate.*

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For this purpose 24 male albino rats of Wistar Institute strain were used. Of these 12 animals were treated (test group) and 12 served as untreated controls. All animals were kept under similar conditions, Purina Dog Chow used as food, and water were constantly present. In addition, green vegetables were given twice weekly. The treated animals received daily (except Sunday) intraperitoneal injections of 0.05 mg testosterone propionate (Perandren) for 53 days beginning at 26 days of age. Control animals remained uninjected.

Weights were taken at 26 days of age and weekly thereafter. Twenty-four hours after the last injection, *i.e.* at 80 days of age, all animals were anesthetized with ether, their carotid vessels were cut and exitus was allowed to result from bleeding. Body lengths measured from the tip of the snout to the anus were then determined.

All data were treated statistically¹ and observed differences between test and control groups were considered as being probably significant only if the "significance ratio" was 3 or more.

* The authors gratefully acknowledge the aid of the Ciba Pharmaceutical Products Company, Inc., for partially defraying the expenses of this study and for furnishing the testosterone propionate (Perandren) used.

¹ Pearl, R., *Medical Biometry and Statistics*, second edition, Saunders, Philadelphia, 1930.

Results. Initial body weights of the control (45.7 ± 2.3 g) and test groups (45.2 ± 1.6 g) showed no significant difference (0.5 ± 2.8 g). The observed difference of 19.7 ± 4.7 g between the final body weights of the test (193.8 ± 3.8 g) and control (174.1 ± 3.0 g) animals in favor of the treated group, however, was probably significant. Likewise, the difference in final body length of 12.0 ± 3.6 mm in favor of the treated group (203 ± 2.8 mm) as compared to the controls (191 ± 2.7 mm) was also probably significant.

The significant increase in body weight and length of animals treated with small doses of testosterone propionate stands in contrast to the growth-inhibiting influence of large doses of this same hormone.² A statement concerning the effect on length is included although initial body lengths were not taken. This was deemed permissible since the test and control animals had originally shown no significant difference in body weight. Body lengths, which are normally so highly correlated with body weights³ may therefore be assumed to have been approximately similar before treatment was begun. The gain in body weight of approximately 11% may not appear very large but when one recalls that growth curves of animals treated with substances lacking growth-stimulating properties remain essentially parallel⁴ and the difficulties encountered in trying to stimulate growth during the early age period of the albino rat,⁵ any gain must be considered seriously.

Conclusions. Testosterone propionate administered intraperitoneally to male albino rats in doses of 0.05 mg daily (except Sunday) from 26 to 80 days of age led to a probably significant increase in body weight and length. This growth-stimulating effect of small doses of testosterone propionate stands in contrast to the growth-depressing effect of large doses of the same hormone.

² Rubinstein, H. S., Kurland, A. A., and Goodwin, M., *Endocrinology*, 1939, **25**, 724.

³ Donaldson, H. H., *The Rat*, Memoirs of the Wistar Institute of Anatomy and Biology, Philadelphia, 1924.

⁴ Rubinstein, H. S., *J. Comp. Neur.*, 1936, **64**, 3.

⁵ Rubinstein, H. S., *Bull. Sch. of Med., University of Maryland*, 1933, **17**, 163.

Action of Gramicidin on Streptococci of Bovine Mastitis.

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Gramicidin—an alcohol-soluble, water-insoluble substance isolated from cultures of a sporulating bacillus—has been shown to exert a marked bactericidal effect against gram-positive microorganisms, both *in vitro* and *in vivo*. It has been found for instance that 0.002 mg of this substance injected intraabdominally into white mice, exerts a therapeutic action against experimental peritonitis caused by pneumococci and streptococci; gramicidin, however, has proved almost completely ineffective when administered by the intravenous, intramuscular, or subcutaneous route.¹⁻³

It is known that in the chronic form of bovine mastitis caused by *Streptococcus agalactiae* (Lancefield group B), the infection is confined to the infected quarter of the udder and rarely results in a demonstrable systemic disturbance. It appeared of interest, therefore, to determine whether gramicidin, when injected into the infected quarter, would destroy the streptococci causing the mastitis.

A number of cases of chronic mastitis were selected for study and it was established by daily bacteriological examination of the milk that the numbers of streptococci remained high (over 100,000 per cc of milk) during a period of several weeks prior to treatment.

The toxic reactions which result from the injection of gramicidin into the bovine udder and a convenient method of administration of the substance, were determined on 2 cows suffering from chronic mastitis. These animals received repeated treatments with increasing amounts of gramicidin diluted in Ringer's solution, which proved very irritating, and later in distilled water, which was more satisfactory. The following technic was finally adopted for the treatment of each individual quarter. Gramicidin in amounts of 60 to 240 mg was diluted in 1000 cc of double distilled sterile water at 40° C. Following the morning milking, the residual milk in the cistern and in the teat was flushed out with 100 to 200 cc of a dilute solution of gramicidin; 800 to 900 cc of the preparation were then

¹ Dubos, R. J., *J. Exp. Med.*, 1939, **70**, 11.

² Dubos, R. J., and Cattaneo, C., *J. Exp. Med.*, 1939, **70**, 249.

³ Hotchkiss, R. D., and Dubos, R. J., *J. Biol. Chem.*, 1940, **132**, 791.

injected under pressure into the quarter and allowed to remain until the next milking. Within one hour after the injection, the treated quarter became distended and the rectal temperature began to increase, reaching 41° C at the 5th or 6th hour. The temperature returned to nearly normal in about 3 hours thereafter, and the acute swelling had about subsided at the next milking.

Repeated treatments of the 2 animals mentioned above failed to eliminate permanently the streptococci from the infected quarters. This may be explained in part by the inadequacy of the method of administration of the bactericidal substance and also by the fact that these 2 animals were well advanced in their lactation periods and that the infected quarters were severely indurated.

Three cows less advanced in the lactation period were selected for the following tests. Nine infected quarters were treated, one of which had been inoculated artificially and allowed to carry an infection for 17 days before treatment. Repeated treatments failed to eliminate the streptococci from 2 quarters. Five treatments were required to sterilize one of the quarters which was moderately indurated. These repeated treatments stimulated the production of fibrosis and resulted in a decrease in milk-secretion. The streptococci disappeared from the other 6 quarters (in 5 cases after a single treatment) without an appreciable decrease in milk production. The fact that streptococci had been eliminated was established by daily bacteriological examination of the milk over periods ranging from 15 to 81 days.

Before the effectiveness of gramicidin in the control of bovine mastitis can be determined, a larger number of animals must be treated and observed over a longer period of time. The influence of fibrosis, the state of the lactation, the competency of the closing mechanism of the teat, and other factors will have to be considered. While the streptococci were not eliminated from all of the treated quarters, they were markedly decreased after each treatment, and the findings thus confirm the results obtained in mice, namely, that gramicidin, when injected directly into an infected focus, exhibits a definite bactericidal effect against streptococci.

A Comparison of Interstitial Cell-Stimulating, Ovarian-Stimulating, and Inhibiting Actions of Pituitary Glands of Different Species.

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There is very little data concerning the luteinizing hormone content of various kinds of pituitaries which are used in experimental work. It has not been accurately established in what amounts the gonad-stimulating hormones exist in the pituitaries of different species of animals. Recently a method of assay has been described which permits the accurate determination of the LH present in pituitary tissue in the presence of the other gonadotropic factor FSH.¹ In most assay methods the FSH acts synergistically with LH, and thus confuses the results.

Likewise, there are no data available regarding the capacity of pituitary glands from different species to inhibit the action of FSH in producing follicular development in the ovaries of immature rats. It has been variously reported (a) that this factor is separate and distinct from the follicle-stimulating and the luteinizing hormone^{2, 3} and (b) that it is the luteinizing hormone which produces this effect under the proper conditions.^{4, 5}

This paper reports the quantitative assay of the LH content and also the inhibiting action of the pituitary glands of sheep, hog, and beef. The potencies of these pituitary tissues in stimulating ovarian development in the immature female rat are also recorded to give a comparative idea of the FSH potency.

Methods of Assay. The increase in the weight of the seminal vesicles of immature male rats has been used as a measure of the luteinizing hormone, since it has been shown that the LH stimulates the production of male hormone in the male rat.⁶ It was also shown that FSH augments the action of LH in the production of male hormone,⁶ so that in its presence the results were not a true measure

¹ Fevold, H. L., *J. Biol. Chem.*, 1939, **128**, 83.

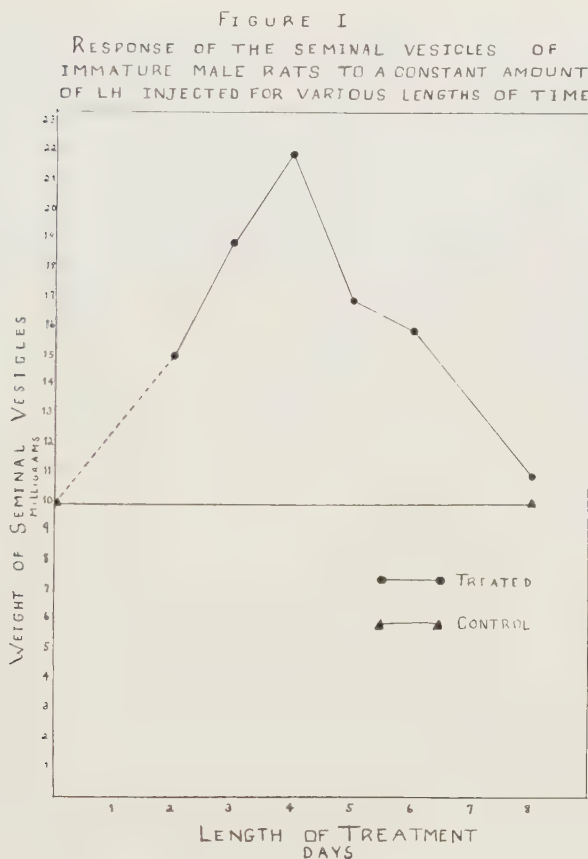
² Evans, H. M., Korpi, K., Pencharz, R. I., and Simpson, M. E., *Univ. Calif., Pub. Anat.*, 1936, **1**, 237.

³ Bunde, C. A., and Hellbaum, A. A., *Am. J. Physiol.*, 1939, **125**, 290.

⁴ Jensen, H., Simpson, M. E., Tolksdorf, S., and Evans, H. M., *Endocrinology*, 1939, **25**, 57.

⁵ Fevold, H. L., and Fiske, V. M., *Endocrinology*, 1939, **24**, 823.

⁶ Greep, R. O., Fevold, H. L., and Hisaw, F. L., *Anat. Rec.*, 1936, **65**, 261.



of the LH. However, if the pituitary material is injected intraperitoneally, FSH is no longer effective, while LH is as active as when injected subcutaneously. It is thus possible to negate the augmenting action of FSH, and the LH activity can be accurately determined.

The pituitary extract was injected intraperitoneally twice daily, morning and evening, in 0.25 cc doses into immature male rats (21 days old) for 4 days, since it was found that injections of a constant amount of LH given for this length of time resulted in the maximum response of the seminal vesicles. (Fig. I.)

A unit of LH is taken as the smallest amount of pituitary tissue necessary to produce a 100% increase in the weight of the seminal vesicles of the injected animals over those of the uninjected controls.

The inhibiting potency of the pituitary glands was determined in the following manner. Enough FSH was given subcutaneously over a period of 4 days to immature female rats (21 days old) to

produce an increase of 300% in the weights of the ovaries. The pituitary preparations to be tested were injected simultaneously intraperitoneally and an inhibiting unit was taken as that amount of pituitary material which would reduce the response to 150%.

The ovarian-stimulating potency was determined by injecting the preparations twice daily for 4 days into immature female rats (21 days old) and weighing the ovaries the morning of the fifth day. At the time of autopsy the ovaries were also observed to determine if they were primarily follicular or luteinized.

Preparation of Materials to be Tested. Acetone-desiccated pituitary powders or fresh pituitary tissue were extracted with an alkaline solvent at pH 8.0, and the extractives precipitated with acetone. The precipitate was thoroughly extracted with distilled water to remove the gonadotropic hormones, leaving as a residue the material rendered insoluble by the acetone precipitation, apparently because of denaturization. The aqueous extracts were then dried and stored as powders. This water-soluble material was always precipitated with tannic acid before injections, and injected as fine aqueous emulsions. In this manner the absorption rate should be as nearly equal from preparation to preparation, irrespective of the impurities, thereby avoiding one source of error.

Results. Table I presents the results of the assay of pituitaries of different species for the luteinizing hormone. It is at once apparent that sheep pituitary glands are the best source of LH and that beef pituitary glands contain the least. With the hog pituitary materials the results vary considerably, but in all cases the LH content is lower than in those of sheep. In 2 of the hog preparations very small amounts of LH were present, as indicated not only by the inability of these 2 preparations to stimulate male hormone secretion but also by the fact that the ovarian development, produced by these

TABLE I.
Interstitial Cell-Stimulating, Ovarian-Stimulating, and Inhibiting-Actions of
Pituitary Tissue of Sheep, Hog and Beef.

Preparation	LH content, Ru/Kg	Ovarian development, Ru/Kg	Inhibiting action, Ru/Kg	Ratios	
				LH/ov.	Lh/inhib.
Sheep pit. powder	143,000	16,000		9.0	
Fresh sheep pit.	20,000	20,000		1.0	
" " "	30,000	10,000	120,000	3.0	0.25
Hog pit. powder	2,000	12,500		0.16	
Fresh hog pit.	1,666	20,000		0.08	
" " "	5,000	15,000	20,000	0.33	0.25
" beef "	1,666	625		2.6	
" " "	2,000	666	10,000	1.6	0.2

preparations, was mainly follicular, with very little luteinization. The third hog preparation contained considerable amounts of LH. It is possible that this variation may be due to a difference in the physiological state of the animals being slaughtered at the time the various batches of pituitary glands were being collected, for it is well known that the pituitary glands of castrated animals contain less LH than do those of normal individuals.

The inhibiting action of sheep, hog and beef parallel their LH content, and the ratio of LH units to inhibiting units was a constant. This would lend support to the belief that the inhibiting property of pituitary extracts may be due to the luteinizing hormone.^{4, 5}

Hog and sheep pituitary glands are approximately equally active in producing ovarian enlargement. This does not mean, however, that hog and sheep glands are equal in FSH potency, but rather that those of the hog have more FSH than those of sheep. This is indicated because there is more LH present in sheep pituitary preparations than in those of hogs. Ovarian development produced by unfractionated extracts is due to the interaction of FSH and LH. Consequently more FSH must be present in hog pituitary glands with the relative small amount of LH in order to produce the same ovarian enlargement as is produced with sheep preparations, which are rich in LH. The ovaries produced with sheep preparations were always heavily luteinized while those elicited by the injection of hog substance were mainly follicular.

Beef pituitary glands produce very little ovarian development and are therefore a poor source of FSH as well as LH.

Summary. (1) It was found that sheep pituitary glands contain the greatest amount of LH, while those from cattle had very little. Hog pituitary glands showed great variation with respect to LH content but in all cases contained much less than sheep glands. (2) The inhibiting action of the pituitary preparations paralleled their LH content. (3) Hog and sheep pituitary glands are approximately equal in producing ovarian hypertrophy. Hog preparations produced mainly follicular development while those of sheep caused the development of heavily luteinized ovaries. Hog glands, therefore, contain more FSH than those of sheep. Beef pituitary glands are a very poor source of FSH. (4) The FSH and LH content of different lots of pituitary glands of the same species varies within wide limits. Nevertheless, those of each species show definite characteristics, with respect to their FSH and LH content.

Effect of Three Synthetic Steroid Compounds upon Weight and Work Performance of Adrenalectomized Rats.*

DWIGHT J. INGLE. (Introduced by F. D. W. Lukens.)

From the George S. Cox Medical Research Institute, University of Pennsylvania, Philadelphia.

The compound 11-desoxy-corticosterone acetate is the most active of the known steroid compounds in respect to its property of maintaining life of adrenalectomized animals. This compound (substance A) may be characterized as pregnene (4:5)-ol(21)-dione (3,20) acetate; its immediate precursor in the laboratory synthesis is (substance B) pregnene (5:6) diol (3,21) one (20) 21-mono-acetate; a third compound, (substance C) pregnene (4:5) triol (17,20,21) one (3), was synthesized by Serini and Logemann.¹ In substance C the stereochemical arrangement at carbon 17 is opposite to that of those steroids occurring in the adrenal cortex which also have a hydroxy group at carbon 17. In these studies substance B was found to possess definite biologic activity although to a much less extent than substance A, and substance C appeared to be inactive in the doses tested.

Male rats of the Sprague Dawley strain which weighed approximately 180 g were used in these experiments. The diet was Purina Dog Chow. Bilateral adrenalectomies were performed in one stage under ether anesthesia. The test substances were dissolved in sesame oil and administered by subcutaneous injection twice daily. The amount of sesame oil injected was kept constant at 1 cc per day for each rat. Ten animals were maintained for 7 days without treatment. Eighty-one rats were treated for 7 days. On the 7th day each animal was weighed and then subjected to the work test. The animals were anesthetized with phenobarbital sodium. The left gastrocnemius muscle was weighted with 100 g and stimulated to contract 3 times per second. Each animal received 5 cc of water twice daily by subcutaneous injection for as long as the animal continued to work. In all of the experiments stimulation was continued

* I wish to express my appreciation to Dr. E. Schwenk, Schering Corporation, Bloomfield, N. J., for the samples of substance A and substance B; and to Dr. R. D. Shaner, The Organon Co., Nutley, N. J., for the sample of substance C.

¹ Serini, A., and Logemann, W., *Berichte der Deutschen Chem. Gesellschaft*, 1938, **71**, 1362.

until the muscle ceased to respond. The details of the method have been described.^{2, 3}

Seven days following adrenalectomy the average body-weight of

TABLE I.

Daily dose mg	Substances					
	pregnene (4:5) ol (21) dione (3,20) acetate		pregnene (5:6) diol (3,21) one (20) acetate		pregnene (4:5) triol (17,20,21) one (3)	
	Wt, g		Wt, g		Wt, g	
	Work		Work		Work	
0.01	176	1543				
	180	2251				
	181	1525				
	174	1221				
0.03	186	3243	153	551		
	195	1810	172	1292		
	194	1971	152	1209		
	182	3532	167	613		
0.06	196	3745	140	dead		
	198	1929	176	1385		
	204	3283	166	882		
	198	1743	170	1410		
0.12	204	3050	178	2187		
	196	3984	162	1606		
	193	4152	178	2770		
	189	5956	177	2074		
0.25	210	5624	183	2263		
	214	4090	182	26		
	188	6695	183	2366		
	206	10024	196	2630		
0.50	216	3392	200	3910	139	16
	204	2405	187	2234	158	857
	186	5725	202	3085	170	830
	191	6890	209	3096	153	1145
1.00	198	8059	186	2586	137	463
	183	6024	205	3464	152	1082
	190	9622	220	3596	153	1467
	200	10355	207	3684	178	1673
2.00	222	18494	211	3496	145	924
	197	6645	204	2812	160	1781
	200	13289	209	4291	165	921
	212	11371	188	4490	157	625
5.00	200	12019	211	3496	154	756
	196	11149	204	2812		
	198	12806	209	4291		
	203	3935	188	4490		

² Heron, W. T., Hales, W. M., and Ingle, D. J., *Am. J. Physiol.*, 1934, **110**, 357.

³ Ingle, D. J., *Am. J. Physiol.*, 1936, **116**, 622.

the 10 untreated rats was 150 g with a range of 134-163; the amounts of work performed averaged 1276 recorder revolutions with a range of 56-2399. Each recorder revolution is equivalent to approximately 400 g-cm of work. The values for body-weight and for work of the treated animals are presented in Table I.

As evidenced by the effect of these substances upon body-weight and upon work performance, the presence of a hydroxy group instead of a keto group on carbon 3 of the pregnene nucleus decreases but does not destroy these biologic effects of the compound. This compound was reported by Waterman and co-workers⁴ to maintain the health of adrenalectomized dogs. The alteration of the molecule to the structure of substance C brought a still greater loss of activity so that substance C appeared to be biologically inactive in these tests. Earlier studies^{5, 6} have demonstrated that although the work performance of adrenalectomized rats treated with substance A is improved over that of untreated animals, it remains very small as compared to sham operated animals. Similar values for work performance of animals treated with substance A were obtained in this study.

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Inhibition of Estrin-Deprivation Bleeding in Rhesus Monkey with Testosterone Derivatives Variously Administered.*

A. R. ABARBANEL. (Introduced by Carl G. Hartman.)

From Morrisania Hospital, Bronx, New York City.

Testosterone and its acetic and propionic acid esters have been shown to inhibit uterine bleeding in the castrate macaque primed with estrogens.¹ In the present experiments a similar effect was attained with methyl-testosterone and ethinyl-testosterone (pregneninolone) and with testosterone di-propionate administered in

⁴ Waterman, L., Danby, M., Gaarenstroom, J. H., Spanhoff, R. W., and Uylert, I. E., *Acta Brevia Neerlandica*, 1939, **9**, 75.

⁵ Ingle, D. J., *Endocrinology*, 1940, **26**, 472.

⁶ Ingle, D. J., *Endocrinology*, in press.

* The writer as well as the staff of the Carnegie Laboratory of Embryology whose hospitality the writer enjoyed, acknowledges with thanks the generosity of the Ciba Corporation for the generous supply of the testosterone compounds and to E. R. Squibb and Sons for keeping the laboratory supplied with Amniotin.

¹ Hartman, C. G., *PROC. SOC. EXP. BIOL. AND MED.*, 1937, **37**, 87.

sesame oil. Some success also followed the oral administration of testosterone propionate given with bile salts, and of methyl and ethinyl testosterone. These experiments were carried out in the rhesus colony of the Carnegie Laboratory of Embryology, Baltimore, Maryland, in the spring of 1939.

1. *Testosterone dipropionate*. 5 mg daily injected into castrated monkey No. 584 for 16 days (June 12-27), after duly priming with estrogen (Amniotin-Squibb), inhibited bleeding and produced the usual² moderate proliferation of the endometrium, which measured up to 2.5 mm in thickness. The vaginal wall showed a fairly thick Dierks layer.

2. *Methyl Testosterone*. A. Administered parenterally. Monkey No. 596, a castrate, bled April 12, 1939 after injections of stilboestrol and was re-primed with the usual 100 R.U. of Amniotin for 9 days (April 17-25). From April 25 to May 20 incl., 5 mg of methyl testosterone in sesame oil were injected subcutaneously daily except Sunday; the animal was sacrificed on May 22. The proliferative action of the hormone was mild but bleeding was successfully inhibited.

B. *Hormone pellets placed subcutaneously*. In castrated monkey No. 584, after due priming with estrogen, eight 3 mg pellets of methyl testosterone were implanted subcutaneously at the end of the injections, April 22, 1939; 5 additional pellets on April 29. No bleeding had occurred by May 12 when biopsies were made. Results as in preceding.

C. *Hormone administered orally*. Beginning on the eighteenth day of a non-ovulatory cycle, 10 mg of methyl testosterone were fed to intact monkey No. 628 to see if the hormone might extend the cycle beyond the maximum of 31 days characteristic of this animal. Feeding was continued through day 46 of the cycle and no bleeding had occurred by day 52, when endometrial biopsies were taken. While bleeding was absent, the endometrium showed almost no proliferative activity, not a single mitotic figure being seen. The organ might almost be called atrophic.

3. *Ethinyl Testosterone (pregneninolone, anhydro-oxy-progesterone)*. A. Administered parenterally. Monkey No. 613, a castrate, was primed from May 16-23, 1939, with 100 R.U. of estrogen (Amniotin) daily. From May 24 to June 10 five mg of ethinyl testosterone in sesame oil were injected daily. No bleeding had occurred by June 16 when the animal was sacrificed. In some areas of the well proliferated endometrium hematmata were noted.

² Hartman, C. G., *Endocrinology*, 1940, **26**, 449.

Apparently bleeding was imminent 6 days after the last injection of ethinyl testosterone. Estrogenic effects on uterus, cervix and vagina were marked.

B. *Hormone administered orally.* Castrated female No. 626 was primed the usual way with estrogen. She was then given one 20 mg tablet of ethinyl testosterone by mouth daily from May 24 to June 7. She began to bleed on the 15th day after the last injection of estrogen. She was sacrificed while still bleeding; while bleeding was not prevented it was probably postponed a few days above the usual maximal interval of 10 days following moderate treatment with estrone (Amniotin). Judging from the state of the uterine, cervical and vaginal mucosæ the effect of oral administration proved far less than that attained by one-fourth as large a dose administered subcutaneously.

4. *Testosterone Propionate given orally.* Monkey No. 591 had her endometrium almost totally removed on Jan. 18 and on Mar. 16 she received 50 mg daily of testosterone propionate. A uterine biopsy was made on May 11. She was castrated on June 9, then fed daily for 19 days two 10 mg tablets of testosterone propionate and one 100 mg tablet of bile salts. The bleeding which usually follows castration within 10 days or less did not occur. On the other hand, the endometrium showed no signs whatsoever of proliferation, measuring but 1 mm in thickness. The condition of the vagina and the cervix, likewise, proved that a minimal quantity of the absorbed hormone reached the systemic circulation.

Summary. 1. Testosterone di-propionate prevented estrin-privea bleeding in daily parenteral doses of 5 mg in sesame oil. 2. Methyl testosterone inhibited estrin-privea bleeding when administered subcutaneously in the form of pellets or dissolved in sesame oil. Orally in daily doses of 10 mg, methyl testosterone prevented menstruation but otherwise failed to exert the slightest visible estrogenic effects. 3. Ethinyl testosterone prevented estrin-privea bleeding in the monkey when administered parenterally in doses of 5 mg a day. Given orally, it delayed slightly but did not prevent bleeding in daily doses of 20 mg, with no other estrogenic effects. 4. Testosterone propionate when administered orally in 20 mg doses along with bile salts, prevented estrin-privea bleeding, but otherwise its estrogenic effects proved minimal. 5. It is apparent that oral administration of any of the testosterone derivatives here tested is most uneconomical as compared with parenteral methods.

Deacylation of N⁴-n-Acylsulfanilamides and N⁴-n-Acyl-sulfanilylhydroxamides *in vitro*.*

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(Introduced by P. A. Shaffer.)

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The acyl derivatives of sulfanilamide are of interest because sulfanilamide is in part acetylated in the animal body before excretion,¹ the acetylated form having only slight therapeutic activity. Higher acyl derivatives have nevertheless been shown to possess therapeutic activity comparable to sulfanilamide. Miller, Rock and Moore² synthesized a series of N⁴-n-acylsulfanilamides, the therapeutic activity of which appears to increase with the length of the acyl group, up to 6 carbons, beyond which it falls off rapidly. N⁴-n-acylsulfanilylhydroxamides are found to possess therapeutic activity³ and high bacteriostatic value *in vitro*.⁴ The water solubility of these compounds decreases with lengthening of the carbon chain.

Aberhalden^{5, 6} and Bergman⁷⁻⁹ described a group of enzymes known as acylases which split acylated amino acids. More recently Michel, Bernheim and Bernheim¹⁰ have described an acylase which splits acetanilid. This enzyme, which they believe identical with the earlier described acylase, is found in high concentrations in liver and kidney of dog, cat, rabbit, ox and mouse.

We have studied the deacylation *in vitro* by rat liver of N⁴-n-acylsulfanilamides and the analogous N⁴-n-acylsulfanilylhydroxamides in which the NH₂-group of the sulfonamide is replaced by an

* This investigation was aided by a grant to P. A. Shaffer from the Rockefeller Foundation.

1 Harris, J. S., and Klein, J. R., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 78.

2 Miller, E., Rock, H. J., and Moore, M. L., *J. Am. Chem. Soc.*, 1939, **61**, 1198.

3 Cooper, F. B., Gross, P., and Lewis, M., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 491.

4 Main, E. R., Shinn, L. E., and Mellon, R. R., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 593.

5 Aberhalden, E., and Ehrenwall, E., *Fermentforsch.*, 1931, **12**, 223, 376.

6 Aberhalden, E., and Heumann, J., *Fermentforsch.*, 1931, **12**, 572.

7 Bergman, M., Zervas, L., and Fruton, J. S., *J. Biol. Chem.*, 1935, **111**, 225.

8 Bergman, M., Zervas, L., and Ross, W. F., *J. Biol. Chem.*, 1935, **111**, 245.

9 Bergman, M., and Ross, W. F., *J. Biol. Chem.*, 1935, **111**, 659.

10 Michel, H. O., Bernheim, F., and Bernheim, M. L. C., *J. Pharmacol. Exp. Therap.*, 1937, **61**, 321.

-NHOH group. On the hypothesis that a free N⁴-amino group is essential for activity it seemed important to learn the extent to which these therapeutically active acyl compounds are deacylated in the animal body. Since the analytical method available (determination of free and total sulfanilamide after hydrolysis) does not distinguish other acyl compounds from the acetyl compound appearing in urine, it seemed preferable to study the hydrolysis by tissue brei with which acetylation does not occur to confuse the results.

Methods. Extracts of liver tissue made by grinding liver with sand and an equal volume of water were strained through cheese cloth. Weights of the acyl compounds equivalent to 1 mg of unacylated compound were added to 20 cc of liver brei representing 5 g of liver in M/20 PO₄ buffer (pH 7.5). Two drops of toluene were added as a preservative. The mixtures were shaken at 37.5°C. Aliquot samples were removed at intervals for analysis. Proteins were removed with 10% trichloroacetic acid (or by alcohol) and filtrates analyzed for free and total sulfanilamide by Marshall's method. Colorimetric determinations were made with an electrophotometer. Percentage of hydrolysis was calculated from the free amine found.

Comparative Hydrolysis of Sulfanilamide Derivatives. We find that the ease and rate of hydrolysis of these acyl sulfanilamide derivatives by liver brei vary with the length of the C-chain in the fatty acid. Acetyl sulfanilamide is decomposed only slowly while long acyl groups are broken off more easily. The ease of hydrolysis parallels the therapeutic activity of these compounds (reported from other laboratories^{2, 3}) which permits the view that the acyl derivatives become active after hydrolysis.

Similar experiments were performed using as substrates acetanilid and 4:4'-acetylamino diphenyl sulfone. Using the same samples of liver brei in tests with the three substrates, acetanilid consistently showed a percentage of hydrolysis higher than that of 4:4'-acetylamino diphenyl sulfone and acetylsulfanilamide.

Tables I, II and III represent the results of typical experiments with N⁴-acyl sulfanilamides, N⁴-n-acylsulfanilylhydroxamides, acetanilid and 4:4'-acetylamino diphenyl sulfone as substrates.

In vivo deacylation of acylated sulfanilamide derivatives has been shown by others from blood and urine analyses. Nitti, Bovet and Hamon¹¹ found that the formyl, acetyl, propionyl and butyryl derivatives of 4:4'-diamino diphenyl sulfone were rapidly hydrolyzed in the body to 4:4'-diamino diphenyl sulfone. Cooper, Gross and

¹¹ Nitti, F., Bovet, D., and Hamon, Y., *Compt. rend. soc. biol.*, 1938, **128**, 26.

TABLE I.
Deacylation of Acylsulfanilamidest by Liver Suspension.

Substrate	% hydrolysis		
	2 hr	5 hr	8 hr
Acetylsulfanilamide	9.1	9.1	8.1
Butyrylsulfanilamide	14.1	27.8	30.4
Valeryl-sulfanilamide	21.7	24.0	25.0
Caproylsulfanilamide	38.3	41.0	41.9
Heptanoylsulfanilamide	63.9	80.1	89.6

TABLE II.
Deacylation of Acylsulfanilylhydroxamidest by Liver Suspension.

Substrate	% hydrolysis		
	2 hr	4 hr	6.5 hr
Acetylsulfanilylhydroxamide	6.6	8.1	9.6
Valeryl-sulfanilylhydroxamide	11.5	15.9	22.7
Caproylsulfanilylhydroxamide	21.8	28.8	41.3
Heptanoylsulfanilylhydroxamide	43.1	62.9	74.5

TABLE III.
Deacylation of Other Compounds by Liver Suspension.

Substrate	% hydrolysis	
	4 hr	8 hr
Acetylsulfanilamidet	5.4	9.4
4:4'-Acetylaminodiphenylsulfonet	10.6	15.0
Acetanilid	58.0	72.0

† These acyl compounds were synthesized and presented to us through the courtesy of Sharp and Dohme, Technical Division, Glenolden, Penn.

‡ These compounds were synthesized and presented to us through the courtesy of Monsanto Chemical Company, St. Louis, Mo.

Lewis³ in their recent study of N⁴-n-acylsulfanilylhydroxamides found that mice given 50 mg oral doses of the valeryl, caproyl and heptanoyl compounds showed approximately 10 mg % of diazotizable material (calculated as sulfanilamide) in the blood 2 hours later.

Conclusions. The ease of deacylation of N⁴-n-acylsulfanilamides and N⁴-n-acylsulfanilylhydroxamides *in vitro* by liver brei is found to increase with the length of the acyl group.

Effect of Aminophyllin, Histaminase,* and Nicotinic Acid on Histamine-Poisoned Puppy Bronchioles.†

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To the large number of drugs recommended for the relief of bronchial asthma, there have been added in the past few years two new ones; aminophyllin (theophylline with ethylene diamine) and histaminase (torantil.) Although marked clinical relief has followed the intravenous injection of aminophyllin in patients with acute bronchial asthma¹⁻³ little experimental work has been done in regard to the mechanism of action of the drug. During the course of this investigation Young and Gilbert⁴ stated that aminophyllin greatly lessens the constricting action of histamine in the smaller bronchi and bronchioles of rabbit lung sections.

Histaminase, while less well established than aminophyllin in the treatment of bronchial asthma, is thought to inactivate any histamine implicated in acute attacks of asthma.

Nicotinic acid has been observed to benefit a few patients at John Sealy Hospital during status asthmaticus⁵ and was therefore included in this study.

Method. Bronchioles were prepared for microscopic observation by the method of Sollmann and Gilbert.⁶ Puppies were killed by the injection of air into the left ventricle. The lungs were removed and injected intratracheally with a warm solution of 10% gelatin in Ringer's solution. They were then placed in iced Ringer's solution for several hours to harden. Thin sections of lung were made free-hand with a razor, and mounted on a ring of cork in a Petri dish containing 50 cc of Ringer's solution. The dish was placed on the warm stage of a microscope, and the solution was kept at a temperature of 38°C. The size of the bronchiolar lumen was recorded by use of a camera lucida.

* Histaminase was generously supplied by the Winthrop Company.

† A preliminary report of this work was published in *Bull. John Sealy Hospital and University of Texas Medical School*, 1940, **2**, 55.

¹ Herrmann, G. R., and Aynesworth, M. B., *J. Lab. and Clin. Med.*, 1937, **23**, 135.

² Efron, J., *Allergy*, 1936, **7**, 249.

³ Brown, G. T., *J. Allergy*, 1938, **10**, 64.

⁴ Young, R. H., and Gilbert, R. P., *J. Am. Med. Assn.*, 1940, **114**, 522.

⁵ Creel, W. F., personal communication.

⁶ Sollmann, T., and Gilbert, A. J., *J. Pharm. and Exp. Therap.*, 1937, **61**, 272.

The addition of 3 mg of histamine acid phosphate usually resulted in complete closure of the bronchiolar lumen. The preparation was observed for about 10 minutes in order to rule out spontaneous relaxation, and then the test drug was added. Nicotinic acid was used in the form of sodium nicotinate, since acid *per se* has a dilator action.⁶

Results. Typical results with aminophyllin, histaminase, and sodium nicotinate are recorded in Table I.

TABLE I.

Puppy No.	Area of normal Bronchiolar lumen, mm ²	Area after histamine	"Dilator" drug applied	Area after "dilator" drug	% of normal area
2	.474	.000	Aminophyllin 1:2000	.336	75
3	.270	.000	" 1:1000	.270	100
2	.456	*	" 1:1000	.558	122
5	.048	.000	Histaminase 4 units	.012	25
4	.216	.000	" 4 units	.000	0
4	.072	.000	Sod. nicotinate 1:1400	.000	0
5	.021	.000	" 1:2100	.000	0

*Histamine not applied.

Aminophyllin 1:2000 to 1:1000 caused marked dilatation of histamine-poisoned puppy bronchioles in all 6 experiments in which it was tried. With normal bronchioles it caused moderate dilatation in 2, and had no effect in one experiment.

Histaminase caused slight dilatation of histamine-poisoned bronchioles in 3 experiments and had no effect in 2 experiments. This slight action after a few minutes was not unexpected, since one unit of histaminase is assayed to neutralize 1 mg of histamine dihydrochloride only after 24 hours' incubation at 37.5°C. Histamine was inactivated by histaminase when the pH was maintained at 7.4 by phosphate buffer during 24 hours' incubation at 37.5°C. Only by fulfilling these rigid conditions was it possible to obtain inactivation.

Nicotinic acid as sodium nicotinate 1:4200 to 1:1400 caused slight dilatation of histamine-poisoned bronchioles in two experiments and had no effect in 8 experiments.

Conclusions. Aminophyllin is an effective dilator of histamine-poisoned puppy bronchiolar sections. Histaminase and nicotinic acid had little or no effect in the concentrations used.

11491 P

Testosterone Propionate, a Bisexual Hormone in the American Chameleon.

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The androgen, testosterone propionate, is known to have some estrogenic effect in mammals;¹ in reptiles this effect is greater. It enlarges the oviduct of the immature alligator² and the adult lizard *Sceloporus*,³ in the latter causing a growth of the mucous glands similar to that produced by theelin. In *Anolis carolinensis*, it will hypertrophy both male and female genital ducts and induce both male and female sex behavior.

Pellets of crystalline testosterone propionate (Ciba)* were implanted subcutaneously into gonadectomized and intact immatures of both sexes and into similar adults. Each category was composed of 4 experimentals and 4 controls occupying the same cage.[†] Immatures received an average of 2.68 mg, of which approximately 1.58 mg was absorbed in 24 days. Adults received an average of 5.22 mg of which 3.03 mg was absorbed in 30-36 days. A group of adult gonadectomized males and another of females were also implanted with pellets of crystalline estradiol dipropionate, averaging 8.50 mg, with absorption of approximately 1.04 mg in 17 days.

The oviducts of ovariectomized and intact immature and adult females were markedly hypertrophied by the pellets. Oviducts of adult ovariectomized controls averaged 6.79 mg while treated females averaged 36.34 mg. In cross-section, their mucosa exhibited numerous glands similar to those produced with estradiol dipropionate. Testosterone-treated immature females showed the same glandular hyperplasia of the mucosa.

Both testosterone and estradiol produced an intense keratinization of the cloaca in all treated females. Dantchakoff⁴ has described this

¹ Groome, J. R., *Quart. J. Exp. Physiol.*, 1939, **29**, 367.

² Forbes, T. R., *Anat. Rec.*, 1938, **72**, 87.

³ Gorbman, A., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 811.

* The authors are indebted to the Ciba Pharmaceutical Products, Inc., for the testosterone propionate (Perandren) and the estradiol dipropionate utilized in this study.

[†] Assistance in the preparation of these materials was furnished by the personnel of Works Progress Administration Official Project No. 65-1-97-23 (WP. 10).

⁴ Dantchakoff, V., *Compt. rend. Soc. biol.*, 1938, **128**, 895.

effect in the *Lacerta* embryos of both sexes following folliculin treatment. The cloacal lining is mucoid in spayed and out-of-season females and also in all males. Testosterone will keratinize the cloacas of castrate immature and adult males. This estrogenic effect was also produced by estradiol in adult castrate males.

Testosterone propionate strikingly enlarges the ovaries of both immature and adult females. Ovaries of the immature females were as much as 3 times the size of controls. Normally only one egg enlarges in each adult ovary at one time. In treated adults, 2 or more ova developed together in one ovary. Weights of ovaries of adult controls ranged from 3.35 mg to 65.63 mg. Ovaries of treated adult females ranged from 31.29 to 309 mg. Testes, however, were smaller than in controls and showed little evidence of active spermatogenesis.

Wolffian ducts, which were very rudimentary in the immature females, were greatly hypertrophied by the testosterone pellets. They became as large as those of treated immature males. The epididymis and ductus deferens of adult castrate males were maintained by testosterone but not by estradiol dipropionate. The latter result is surprising since theelin will produce an enlargement of the male ducts in adult *Eumeces*⁵ and young *Anolis*.⁶

Certain tubules of the kidney were markedly hypertrophied in all testosterone-treated *Anolis*. This "sexual segment" of Regaud and Policard⁷ is in secretory activity in the normal adult males and assumes this condition in all testosterone-treated males and females. Kehl⁸ reported this effect with benzoate of androsterone in adult female *Uromastix*. Gonadectomized control and estradiol-treated *Anolis* showed uniformly small kidney tubules.

Testosterone propionate pellets will induce male courtship and copulation in immature and adult females, whether ovariectomized or intact. The same implanted females will show estrous behavior and may be copulated with by either males or treated females. Estrous behavior includes: (1) a distinctive bend of neck, and (2) voluntary submission to copulation. Testosterone-treated females were observed to stand with necks flexed in this manner before males and treated females.

Pellets of testosterone propionate increase the aggressiveness of females. One treated female eventually dominates the group and assumes the rôle of a territory-holding male. Her activity partially

5 Turner, C. D., *Biol. Bull.*, 1935, **69**, 143.

6 Clapp, M. L., *Anat. Rec.*, 1937, **70** (Suppl. 1), 97.

7 Regaud, C., and Policard, A., *Compt. rend. Soc. biol.*, 1903, **55**, 973.

8 Kehl, R., *Compt. rend. Soc. biol.*, 1938, **127**, 142.

inhibits that of the other treated females. These, however, may show male behavior and also submit to copulation. Adult males, through their larger size and aggressiveness, are able to subdue the most dominant treated female which then submits to copulation.

Testosterone propionate pellets also produce full sex activity in immature and adult castrate males. One treated immature male, on 3 separate occasions, exhibited the estrous bend of neck and was copulated with 5 times. This male likewise copulated twice in male manner.

Summary. Testosterone propionate enlarges the Müllerian duct, keratinizes the cloaca and produces estrous behavior in *Anolis*. It also enlarges the epididymis, ductus deferens and sexual segment of the kidney, while producing male sex behavior. It has a gonadotropic effect on the ovary but not on the testis.

11492 P

Size and Stroke of the Normal Human Heart During Neosynephrin Bradycardia.*

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Marked bradycardia with pulse rates from 30 to 50 per minute is produced in normal young adults by therapeutic doses (3 to 10 mg subcutaneously) of neosynephrin—1- α -hydroxy- β -methylamino-3 hydroxy ethylbenzene hydrochloride (Keys and Violante¹). The effect persists for 30 to 60 minutes or more and is not attended by any symptoms or sensations of cardiac or respiratory embarrassment. Since repeated trials failed to disclose any significant change in the total oxygen usage during the bradycardia it seemed probable that the total minute output of the heart was not seriously diminished. If this were so there should be a very appreciable increase in the stroke output. We have investigated this question with the roentgenkymographic method of Keys and Friedell.²

* This work has been supported by a Fellowship grant to the Laboratory of Physiological Hygiene of the University of Minnesota by Frederick Stearns and Co.

¹ Keys, Ance!, and Violante, Antonio, PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 4.

² Keys, Ance!, and Friedell, H. L., *Am. J. Physiol.*, 1939, **126**, 741.

Trained normal young men and women subjects were used. All studies were made in the post-absorptive state in the early morning with the subject seated in a roller chair in a quiet room. When a roentgenkymographic exposure (R.K.G.) was made (66 inches), the chair was rolled into position and the subject coöperated only to the extent of holding the breath during the 1.5 second exposure. After a preliminary rest of 20 minutes or more, one or 2 R.K.G.s were made before injection of the drug. R.K.G.s were made subsequently when the bradycardia was well established—usually 10 or 15 minutes later—and when the bradycardia had begun to diminish. In some cases a final R.K.G. was made when the pulse and blood pressure were nearly normal again.

A striking alteration of the heart size was frequently apparent even from casual inspection of the resulting films. When the areas of the anterior-posterior projections were measured it was found that there is an increase of 5 to 20% or more during the period of bradycardia. The change is more remarkable when the corresponding volumes are calculated from our formula (op. cit.): $\text{vol.} = 0.63 (\text{Area})^{1.45}$. To illustrate, we may cite 3 cases, selected at random, and compare the diastolic volumes in cc before and 15 minutes after subcutaneous injection of 5 mg of neosynephrin:

	Subj. E.H.	Subj. D.W.	Subj. B.N.
Before	467	503	506
After	497	601	625

The left side of the heart shows the most pronounced increase in size but all parts of the heart appear to share in the dilatation and the original form of the heart is well preserved in both systole and diastole. We have never observed any signs of pericardial restraint, in spite of the fact that the dilatation in many cases surpasses what is frequently considered to be the upper limit for immediate dilatation. In a number of cases the P.A. transverse diameter increased more than 15 mm; in one case the increase was 19 mm and in another 18 mm. In all cases the degree of inspiration was the same.

Significant increases in diastolic heart size were found in 90% of all our studies with neosynephrin. It does not appear when epinephrine or sterile saline are similarly administered. The systolic volume of the heart also increases, but to a lesser extent, so that there is a definite and usually large increase in stroke volume. In 12 studies on 8 subjects the mean stroke volume before injection was 57.5 cc; between 15 and 30 minutes after injection of neosynephrin the mean indicated stroke volume was 90.1 cc. The net effect is usually to leave the minute volume relatively constant though there may be a slight reduction in the minute volume after the largest doses

(10 mg) and a net increase in minute volume frequently results from a rather small (3 to 5 mg) dose of the drug.

The contraction form shown by the R.K.G. corresponds to the RT interval of the E.C.G. in that ventricular contraction and discharge are not unduly prolonged. It is notable, however, that during much of diastole the left ventricle appears to pause at constant volume (diastasis). The R.K.G. film usually resembles extreme athletic bradycardia.

These studies are being continued with additional techniques. The first few experiments with the acetylene method have shown an increased stroke volume of the same general magnitude found with the R.K.G. method, so that apparently the dilatation does not invalidate the volume calculations.

11493 P

Effect of Relative Humidity on Insensible Weight Loss of the Newborn Infant.*

JOHN A. ANDERSON. (Introduced by Irvine McQuarrie.)

From the Department of Pediatrics, University of Minnesota, Minneapolis.

Although Rubner and von Lewschew¹ and Benedict and Carpenter² noted that high relative humidity of the environmental air produced a decrease in the rate of insensible weight loss in both man and experimental animals, recent investigators^{3, 4} have stated that, within the range of average environmental conditions, the relative humidity produces no significant effect. The failure of the insensible weight loss method in predicting accurately the energy metabolism of infants prompted this investigation of the effect of changes in relative humidity on the insensible weight loss. This report deals with 76 observations on the insensible weight loss in 41 unclothed newborn male infants at relative humidities ranging from 19% to 94% and at an environmental temperature of 31.2-32.5°C (87-89°F).

* This study was made possible by a grant-in-aid from Mead Johnson and Company of Evansville, Indiana.

¹ Rubner, M., and von Lewschew, *Arch. f. Hyg.*, 1897, **29**, 1.

² Benedict, F. G., and Carpenter, T. M., Carnegie Inst., Washington, 1910, Pub. No. 129.

³ Levine, S. Z., Wilson, J. R., and Kelly, M., *Am. J. Dis. Child.*, 1929, **37**, 791.

⁴ Yaglou, C. P., *J. A. M. A.*, 1937, **108**, 1708.

Each experiment was carried out under the following conditions: Thirty to 45 minutes after breast feeding, the infant was placed in an accurately controlled air-conditioned chamber[†] and weighed by means of a Sauter scale for 30 to 90 minutes. A condition of sleep and absence of sweating were required before measurements were made.

The results are summarized in Table I.

TABLE I.
Average Insensible Weight Loss at Various Humidities.

Case No.	% relative humidity		G insensible wt loss per hr		
	Range	Avg	Infant	Kg	Sq.M.
11	19-30	21.6	3.82	1.16	17.2
16	31-40	35.8	3.24	.94	13.7
5	41-50	48.5	2.67	.86	12.3
19	51-60	54.0	2.58	.80	11.7
8	61-70	65.0	1.99	.60	8.8
11	71-80	76.5	1.35	.39	4.7
6	81-94	88.0	1.19	.40	5.0

The average insensible weight loss for each 10% increase in relative humidity decreases in a straight line manner to a negligible value at 100% relative humidity. The generally accepted value⁵ for insensible weight loss of 1.0 g per kg per hour for infants falls between 25% and 35% relative humidity. This value is approximately twice that observed at 60% and 70% relative humidity. The general slope of the line indicates that for each 10% increase in relative humidity within the range studied, there is 0.46 g decrease in the hourly rate of insensible weight loss for the average newborn infant. From these data it appears that attempts to predict the energy metabolism of the newborn infant from the insensible weight loss according to the formulas of Benedict and Root for adults⁶ and Levine and Marples for infants⁷ are not valid unless the effect of the relative humidity is taken into consideration.

Summary. The rate of insensible weight loss in the unclothed, newborn male infant is decreased in a straight line manner by an increase in the relative humidity of the environmental air.

† This unit was constructed with the advice and assistance as well as the donation of special equipment from the Minneapolis Honeywell Regulator Company.

⁵ Levine, S. Z., Kelly, M., and Wilson, J. R., *Am. J. Dis. Child.*, 1930, **39**, 917.

⁶ Benedict, F. G., and Root, H. F., *Arch. Int. Med.*, 1926, **38**, 1.

⁷ Levine, S. Z., and Marples, E., *Am. J. Dis. Child.*, 1930, **40**, 269.

Effect of Relative Humidity on Skin and Rectal Temperatures of the Newborn Infant.*

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In a previous investigation¹ on unclothed newborn, male infants the author showed that an increase in the environmental relative humidity resulted in a decrease in the rate of insensible weight loss. In an attempt to explain the physiologic processes involved in the adjustment of the heat loss mechanism of the body induced by high humidity, measurements of the surface and rectal temperatures were made under similar experimental conditions. The skin surface temperatures of the forehead, abdomen, and dorsum of the hand and foot were determined by means of copper constantin thermocouples. A pessor catheter containing a thermocouple was used for obtaining the rectal temperature. The insensible weight loss and the skin and rectal temperatures of twelve unclothed, newborn infants were determined first at a low relative humidity and, after a period of adjustment of 45 to 60 minutes, again at a higher humidity.

The results obtained are presented in Table I.

In the cases in which sweating did not occur (Cases 1-9), for an

TABLE I.
Changes in Skin and Rectal Temperatures Induced by Change in Relative Humidity.

Case No.	Change % relative humidity	Change Temperature degrees C						I.L. change g
		Air	Forehead	Hand	Abdomen	Foot	Rectal	
1	47	.0	.0	+1.7	+0.6	+2.4		-2.06
2	38	+ .1		+2.8	+1.1	+3.2	+.4	-1.65
3	34	+ .8		+1.7	+1.2	+2.0	+.9	-1.72
4	40	+ .3		+0.8	-1.0	+2.5	+.8	-1.35
5	56	+ .8	+ .9	+2.1	+1.0	+4.3		-2.34
6	34	+ .3	+1.0	+2.1	+0.9	+1.6		-1.82
7	26	+ .7		+0.2	+1.6		.0	-0.44
8	25	+ .1		0.0	+0.2	+0.8	.0	-0.56
9	44	+2.2	+ .9	+2.6		+3.5	+.7	-2.76
Avg	38	+0.4	+ .7	+1.5	+0.8	+2.5	+.4	-1.63
Cases in Which Sweating Occurred.								
10	24	.0	+ .1	+1.1	+0.3	.0	.0	+0.04
11	24	+ .2			.0	+2.0	+.2	+1.25
12	46	+1.0	+1.8		+1.7	+4.5	.0	+0.64

* This study was made possible by a grant-in-aid from Mead Johnson and Company of Evansville, Indiana, and by the donation of special equipment, together with advice and assistance, from the Minneapolis Honeywell Regulator Company.

¹ Anderson, J. A., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 464.

average increase of 38% relative humidity there was an average increase in the surface temperature of the body which occurred in the following manner: the foot— 2.5°C , the hand— 1.5°C , the abdomen— 0.8°C , and the forehead— 0.7°C . Accompanying these changes, the average decrease in the rate of insensible weight loss was 1.63 g per hour.

There were two types of response of the skin temperatures of these infants. Six of the infants responded by an increase in the surface temperature of the dorsum of the hand and foot, which occurred within 10 minutes after the change from the low to the high humidity. When the high humidity was maintained at a constant level, an increase in the temperature of the abdomen and forehead occurred if the experiment was prolonged or if the increase in humidity was excessive. At this time all surface temperatures continued to increase uniformly to a level just below that of the rectal temperature, which usually remained constant. The remaining 6 infants had an increase in the surface temperatures of all parts of the body at the same time, which usually occurred within 10 minutes following the increase in relative humidity. These skin temperatures tended to stabilize at a higher level with moderate increases in humidity; or if the humidity increase was excessive, they approached the rectal temperature, which also increased slightly. Detectable sweating then occurred, followed by a fall in rectal temperature to or below the original level. The decrease of the rate of insensible weight loss was noted in both groups up to the time of the occurrence of sweating.

The increase in the surface temperature of the newborn infant induced by increasing the relative humidity of the environmental air offers an explanation of the mechanism involved in the decrease of the insensible weight loss under the same conditions. The systematic manner in which the surface temperatures of the foot, hand, abdomen, and forehead increase with the increase in relative humidity is similar to that reported by Freeman and Lengyel for adult human subjects.²

Summary. In unclothed, newborn male infants the decrease in the rate of insensible weight loss is accompanied by an increase in the surface temperature of the skin at high relative humidity. The vasomotor responses necessary for this adjustment in the heat loss mechanism under these conditions appears to be as fully developed in one-half of the infants studied as in adult subjects.

² Freeman, H., and Lengyel, B. A., *J. Nutrition*, 1939, **17**, 43.

Cultivation of Pleuropneumonia-Like Organisms from Female Genital Organs.*†

L. DIENES.

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The technic formerly described for staining bacterial cultures *in situ* on the surface of agar was employed in studying routine plates submitted for gonococcus examination.¹ In the course of 2 months pleuropneumonia-like organisms were demonstrated in the cervical secretions of 5 patients. The medium used for the gonococcus is similar to the medium employed formerly in cultivating *Streptobacillus moniliformis* and pleuropneumonia-like organisms.¹ It is essentially a sedimented boiled blood agar to which is added 30% buffered ascitic fluid. The plates are incubated for 2 days in partial CO₂ tension.

Pleuropneumonia-like organisms were present in the genitals of about one-third of the females. Thus far similar organisms have not been found in plates inoculated with secretions from the urethra or prostate of males or from eyes of babies suspected of gonococcus infection. However, the female and male material examined was not comparable. The majority of female patients had pelvic infections, while the cultures from males were mostly release cultures from treated gonococcal patients. Women without pelvic disease were not studied.

The group of pleuropneumonia-like organisms is characterized at present by purely morphological criteria. The organisms cultivated from the female genitalia are indistinguishable in morphology and in the appearance of colonies from the strains isolated from rats and mice.² The young colonies consist of very small pleomorphic granules and filaments which grow into the medium and are stained deeply *in situ* with methylene blue. The surface of fully developed colonies consists of large bodies (3 to 10 microns) which are at first deeply stained but which later become vacuolized and produce a foam-like structure. After 48 hours the colonies are often only

* The expenses of this investigation were defrayed in part by a grant from the Commonwealth Fund.

† This is publication Number 47 of the Lovett Memorial.

¹ Dienes, L., *J. Inf. Dis.*, 1939, **65**, 24.

² Dienes, L., and Sullivan, E. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 424.

10 to 20 microns in diameter. In transplant they develop to a considerably larger size. Four strains were isolated in pure culture and their properties will be more closely studied.

The tiny colonies were present in abundance in all cultures except one. In 2 cases they were associated with the gonococcus; in 3 they were found in the absence of gonococcus. In one case they persisted in the cervical smear even though the gonococcus disappeared following the administration of sulfanilamide.

In a previously described case a similar organism was isolated in pure culture from a suppurated Bartholin's gland.³ At that time, it was thought the patient's contact with rats might have been responsible for the infection. With the knowledge that similar organisms occur frequently in the female genitalia it seems more probable that the previously observed suppuration was caused by a human strain and that such strains are potentially pathogens. Unfortunately, the strain isolated from the suppurative lesion was lost, therefore its origin cannot be established. According to Sabin's observations, mice often harbor pleuropneumonia-like organisms in the conjunctiva.⁴ These organisms, although usually harmless, may under appropriate conditions become pathogenic. The pathogenicity of the cattle, goat and rat strains is well known. It is of special interest that all members of the pleuropneumonia group produce acute or chronic joint lesions.

At present, it is impossible to state whether the strains isolated from female genitalia are potentially pathogenic although the above mentioned single observation suggests that they may be. They may only represent another variety of the many unknown saprophytic microorganisms of the mucous membranes. The frequent presence of a member of the pleuropneumonia group of microorganisms in human beings certainly deserves further study.

³ Dienes, L., and Edsall, J., *PROC. SOC. EXP. BIOL. AND MED.*, 1937, **36**, 740.

⁴ Sabin, A. B., *Science*, 1939, **90**, 18.

L Type of Growth in Gonococcus Cultures.*†

L. DIENES.

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It was described in a former note that in cultures of various Gram negative bacteria tiny secondary colonies similar in many respects to the L1 colonies of *Streptobacillus moniliformis* develop.¹ The appearance of these colonies is always preceded by the transformation of the bacteria into large swollen forms. More recently it has been observed that the secondary colonies develop from these large forms.²

A similar course of events was observed in gonococcus cultures. In certain cultures the cocci before disintegrating swell up into large deeply stained spherical bodies similar in every respect to the large bodies of Gram negative bacteria. If such cultures are kept one or two days at a temperature between 25 to 30°C, one notes below the colonies in the agar a slight secondary growth very similar to the L type of growth observed in colon bacillus and influenza colonies. It consists of small granules and fine filaments which usually degenerate in 24 hours. In gonococcus cultures this peculiar secondary growth does not develop as abundantly nor as distinctly as in the cultures of Gram negative bacteria, and without the experience obtained with the latter it would probably have been overlooked. The main evidence in support of the supposition that this slight transient growth corresponds to the development of the L type colonies is the essential similarity of the whole process in different bacterial cultures.

The observation of this process in gonococcus cultures possess some importance inasmuch as it is the first example of the occurrence of this process in a species of bacteria besides the Gram negative bacilli. Attempts to demonstrate a similar secondary growth in the colonies of Gram positive cocci, especially in the colonies of streptococci, have been unsuccessful thus far.

The process described in this note has nothing to do with the presence or absence of pleuropneumonia-like organisms in the cul-

* The expenses of this investigation were defrayed in part by a grant from the Commonwealth Fund.

† This is publication Number 48 of the Lovett Memorial.

¹ Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 636.

² Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 703.

tures. The occurrence of such organisms with the gonococcus has been indicated in a preceding note.³ The pleuropneumonia-like organism grows independently of the gonococcus, and mixed cultures are easily separated. The tiny secondary colonies develop only in connection with the large swollen bacterial forms and thus far all attempts to grow them separate from the parent organism have failed. Morphologically, the secondary growth is very similar to a young growth of the pleuropneumonia-like organism.

11497 P

Developmental Relationship Between Pars Intermedia of Pituitary and Brain in Tadpoles.

WILLIAM ETKIN. (With the assistance of Rose Lotkin.)

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An inhibitory control of the growth and functional activity of the pars intermedia of the hypophysis of the tadpole through the infundibulum has been suggested by the author to account for the finding of overgrowth and excess activity of this gland in grafts, and after infundibular lesion.¹ In his careful work with pituitary grafts in the salamander, however, Blount² reported an intensity of pigmentation only in proportion to the number of grafts and no overgrowth in the grafts. Since in Blount's work successful grafts were secured only when brain was transplanted with the gland, whereas in this author's work with tadpoles the graft took successfully independently of the presence of brain it was thought that the circumstance of the presence of brain with the primordial graft might account for the difference in the characteristic growth picture of the graft in these two cases. This theory would be consistent with the first mentioned hypothesis of inhibitory control of the p. intermedia through the infundibulum. To test this, a series of grafts of the pituitary was made with and without brain.

The experiment was performed on *Rana pipiens* tadpoles. The

³ Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 468.

* Assistance in the preparation of these materials was furnished by Works Projects Administration for the City of New York, Project Number 65-1-97-23 W.P. 10.

¹ Etkin, W., and Rosenberg, L., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 332.

² Blount, R. F., *J. Exp. Zool.*, 1932, **63**, 113.

hosts were tadpoles which had been hypophysectomized in the tail-bud stage and used when about 12 mm total length. Only silver (successfully hypophysectomized) animals were used. The site for the implant was prepared by removing the eyeball through a slit in the dorsal skin, thus leaving a relatively large pocket for the reception of the graft. The grafts were taken from tail-bud embryos. In one series the pituitary primordium with as little adherent tissue as possible was used, and in the second series a variable amount of adjacent brain material was included with each graft. Normal unoperated controls were run simultaneously. When tadpoles were fully grown they were killed and the head serially sectioned.

In the first series of grafts without brain the same experimental types obtained as previously. Thus of a total of 18 surviving experimentals of this type 7 became very much darker than normal and showed on sectioning enlarged p. intermedia grafts with cellular hypertrophy and intense basophilia in the cytoplasm, 2 showed approximately normal pigmentation and on sectioning showed diffuse degenerating grafts with apparently little p. intermedia, 9 remained silver in color and the 3 of these which were sectioned showed no grafts persisting.

In the group receiving grafts with brain and pituitary 32 survived well. Of these 6 showed marked hyperpigmentation. The sections of these animals showed the characteristic hypertrophy and hyperplasia in the p. intermedia. Varying amounts of brain were present in the graft. In no case was the gland in contact with recognizable infundibular tissue though in some it was in contact with other parts of the brain.

Ten animals which fell into the silver class showed on sectioning no clear p. intermedia tissue in the graft. The critical class of experimentals was the group of 15 individuals which showed either normal pigmentation or a moderated degree of hyperpigmentation. Of these 6 proved to have degenerating grafts with doubtful or very scattered p. intermedia and 9 showed normal well formed p. intermedia grafts in which the cells were normal in appearance. Seven of these grafts were directly in contact with recognizable grafted infundibular tissue, one was directly in contact with brain tissue of uncertain form and one was in contact with the infundibulum through the intermediation of p. anterior tissue.

This experiment supports the following interpretation. When p. intermedia develops in contact with the infundibulum its growth and histological development are normal and its functional activity is not much if at all in excess of normal. When, however, it de-

velops separated from the infundibulum its growth is excessive, it shows cellular hypertrophy and produces an excess of the pigmentary hormone which induces intense hyperpigmentation in the host. The infundibulum normally controls p. intermedia function by inhibition.

11498 P

Influence of Age on Rate of Immune Response of Mice to Formolized Equine Encephalomyelitis Virus.

ISABEL M. MORGAN. (Introduced by Peter K. Olitsky.)

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It has been reported¹ that the ability of mice to be immunized with formolized virus of Eastern equine encephalomyelitis increases with age. The question then arose as to whether this was an expression of different rates of development of immune response, or rather of maximum responses of which various age-groups were capable. Rate of antibody response to a trypanosomal infection in rats was found to increase with age.²

The rate of development of neutralizing antibodies in serum of mice of 3 age-groups was studied. Mice 3 months, 14-15 days and 4-5 days of age were injected intraperitoneally on the 1st, 3rd and 5th days with 0.2-0.25 cc of formalin-inactivated virus of Eastern equine encephalomyelitis. This consisted of a 10% suspension of infected mouse brain in 0.5% formalin, which proved to be non-infectious on intracerebral injection of normal mice. Mice in each group were bled from the heart and the sera pooled, on the days indicated in Fig. 1, *i.e.*, 4th, 5th (4 hours after the last injection of formolized virus), 6th day, etc. Serum-neutralizing antibodies were measured by the intraperitoneal protection test³ in normal mice from 13 to 15 days of age, using 4 mice for each virus dilution-serum mixture. Sera of different age-groups taken on the same day were compared simultaneously.

The antibody titer is recorded in Fig. 1 as doses of virus neutralized. This was calculated from the difference between the infective

¹ Morgan, I. M., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 501.

² Kolodny, M. H., *Am. J. Hyg.*, 1940, **31**, 1, Sec. C.

³ Olitsky, P. K., and Harford, C. G., *J. Exp. Med.*, 1938, **68**, 173.

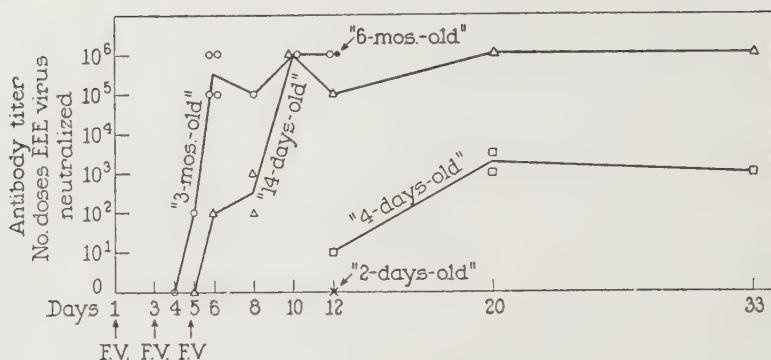


FIG. 1.

Rate of development of neutralizing antibodies in serum of mice of various ages in response to 3 doses of formalized Eastern equine encephalomyelitic virus (F.V.).

titer in control and test groups of mice.* It was found that differences in titer must be more than tenfold to be significant. In serum of mice immunized at 3 months of age, neutralizing antibodies began to appear on the 5th day reaching a maximum by the 6th day. In mice 14-15 days old at the beginning of immunization, antibodies were not demonstrable until the 6th day and reached a maximum between the 8th and 10th days, which was maintained for at least 23 days longer. Considerably slower in response were mice 4-5 days old at the beginning. A minimum of antibody was demonstrable on the 12th day. Sera taken on the 20th and 33rd days protected $\frac{1}{4}$ to $\frac{3}{4}$ of mice in groups receiving a wide range of virus dilutions, indicating moderate antibody content. This is in contrast with sharp endpoints obtained with weak or strong antiserum. Sera of "2-days-" and "6-months-old" mice are also shown. Thus the rate of antibody production was found to increase with age. Below a certain age, the final titer reached depended on the age at which immunization was begun.

In order to determine whether the low grade of active immunity reported previously¹ in young mice would increase with time, 2 large groups of mice, 14 days and 3 months old, were immunized as described. Mice from each age group, as well as 3-months-old normal controls, were tested by intracerebral injection of tenfold dilutions of active virus after 2, 3 and 4 weeks. The "3-months-old" immunized mice resisted 10^7 , 10^5 - 10^6 and 10^6 - 10^7 doses respectively, as measured by difference in titer between control and test groups.

* For example, if the titer of virus in the presence of normal serum were 10^{-7} and with test serum, 10^{-5} , the difference in titer would be 10^2 or 100 doses neutralized.

The "14-days-old" mice were not uniformly resistant; 50% of mice receiving from 1 to 1,000 doses survived. The proportion surviving, of those tested 2, 3 and 4 weeks after immunization, did not increase. It was shown therefore that the high degree of active immunity of "3-months-old" mice was maintained during the 2-4 weeks' period following immunization; and the low degree of resistance of mice immunized when 14 days old did not change significantly during that period, in spite of the observation (Fig. 1) that mice of this age group possessed maximum titer of circulating antibodies.⁴

Summary. The rate of development of neutralizing antibodies in serum of mice immunized with formalin-inactivated virus of Eastern equine encephalomyelitis has been shown to increase progressively with age. The antibodies in serum of mice immunized at a very early age did not reach the maximum titer found in mice immunized when older.

The low degree of active immunity to intracerebral injection of active virus induced in mice 14 days old at the beginning of immunization did not increase from 2 to 4 weeks after immunization. During that interval, mice immunized at 3 months of age maintained a high degree of active immunity.

11499 P

Chronic Histamine Action.*

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(Introduced by M. B. Visscher)

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Histamine placed in the body in watery solution rapidly produces acute effects of relatively short duration (Dale and Laidlaw¹). During the past 2 years studies have been undertaken to develop a procedure by which injected histamine would act over prolonged periods and produce chronic effects. The ultimate aim of the investigation was the study of chronic histamine poisoning.

Experimental Procedure. Histamine was administered by sub-

⁴ Olitsky, P. K., and Harford, C. G., *J. Exp. Med.*, 1938, **68**, 779.

* Part of the expense of this research has been defrayed by grants from the Committee on Scientific Research of the American Medical Association (No. 526 and 556) and the Graduate School of the University of Minnesota.

¹ Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1910, **41**, 318.

cutaneous or intramuscular injection. All doses mentioned are in terms of histamine base. Two tests were employed to determine the efficacy of the methods used to slow the rate of absorption of histamine from the sites of injection. The first was a comparison in normal guinea pigs of the effects of large doses of histamine in saline solution with the effects of the same dose of histamine in other substances. While this method was satisfactory for the preliminary experiments it was inadequate for the quantitative determination of the extent and degree of prolonged histamine action. The second test was the measurement of the amount and quality of the secretion from gastric pouches of dogs made according to the method of Heidenhain. This test provided a quantitative basis for studying the extended action of histamine. The pouches were prepared under ether or nembutal anaesthesia using the usual surgical technic and sufficient time for recovery was allowed before tests were commenced.

Histamine was first ground with glycol stearate and then suspended in mineral oil. In guinea pigs, injection of this material was quickly followed by typical symptoms and fatalities. Similar results were obtained when histamine particles were covered with paraffin and suspended in oil. Definite protection was obtained with a mixture of finely powdered histamine, pure beeswax or beeswax containing resin and mineral oil. Quantities of histamine which caused fatal reactions when given in saline solution produced only mild symptoms when administered with beeswax. Hot saline extraction of this dose of the beeswax mixture gave watery solutions which when injected produced profound reactions or death. Mixture of the histamine with the beeswax had not destroyed the histamine.

The histamine beeswax mixture has been quantitatively tested by the gastric secretion method in 4 dogs. Doses of histamine ranging from 15 to 60 mg were given. As a routine the volume of material injected was about one cubic centimeter and this was divided among approximately 20 intramuscular sites. Reactions were noticed in only 2 out of 10 experiments. As a rule gastric secretion commenced 10-15 minutes after the injection and continued for from 24 to over 40 hours. The total volume of juice secreted in 24 hours expressed as equivalent volume N/10 HCl ranged from 957 to 1919 cc. In the majority of instances the maximum rate of secretion was reached during the first 4 hours following which it declined gradually. The water, chloride and hydrogen ion loss was combated by dilute saline drinking water, saline solution by vein and under the skin and by the return of gastric juice with a stomach tube. In

these experiments prolonged action of a single injection of histamine was obtained by suspending the histamine particles in a beeswax mixture.

11500

Salt After Adrenalectomy. I. Growth and Survival of Adrenalectomized Rats Given Various Levels of NaCl.*

EVELYN ANDERSON, MICHAEL JOSEPH AND VIRGIL HERRING.

(Introduced by Herbert M. Evans.)

From the Institute of Experimental Biology and the Department of Medicine of the University of California, Berkeley and San Francisco, California.

It is well known that the administration of sodium chloride to animals deprived of their adrenals considerably delays the onset of adrenal insufficiency, but the importance of the amount of sodium chloride given such animals has not received adequate attention. There is an "optimal" amount of sodium chloride from which such an animal derives benefit; an excess of sodium chloride is injurious. Moreover, inadequate study has been given the matter of the degree to which functional restoration occurs in adrenalectomized animals given salt. These are the problems which concern us here. This study deals with the growth and survival of adrenalectomized animals in response to varying amounts of sodium chloride. Two succeeding reports deal with the urinary excretion of Na and K and with the storage of fed carbohydrate by such rats.

Adrenalectomized rats fed a standard diet and allowed to drink 1% NaCl take in an amount of salt which appears to be optimal for maintaining growth and health. For this study male rats were used; they were 10 weeks of age at the time of adrenalectomy, and weighed approximately 200 g. Controls were subjected to a sham operation in which the adrenals were dissected free of the surrounding tissue but not removed. From a group of 25 adrenalectomized rats given 1% NaCl solution, 15 had an average survival of 45 days (range 12-80 days) and 10 lived beyond the 110th day post-operative. During the course of the experiment all of the animals were tested for completeness of adrenalectomy by withdrawing salt and allow-

* We wish to acknowledge the assistance of the Federal Works Progress Administration, Project No. OP 65-1-08-62, Unit A-5, and the Christine Breon Fund.

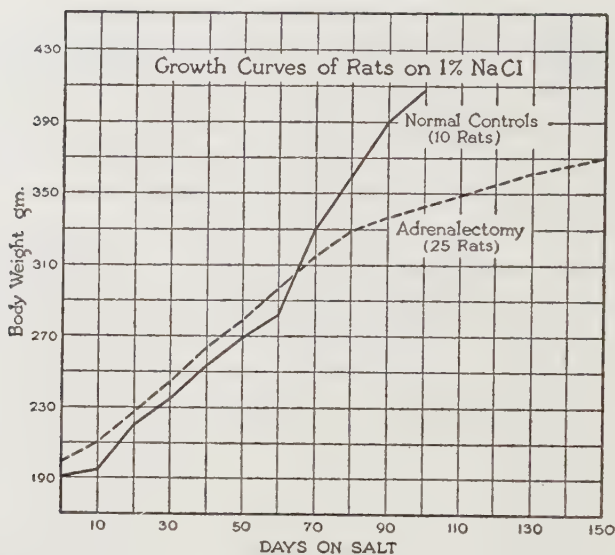


CHART I.

Showing the growth response of adrenalectomized rats given 1% NaCl to drink.

ing the animals to go into a state of adrenal insufficiency. The average daily intake of sodium in food and drinking water was estimated to be about 290 mg (725 mg NaCl). The growth curve of these animals is shown in Chart I. The adrenalectomized rats on 1% NaCl continued to grow at the same rate as the control animals for about 2 months; after that the growth rate was diminished. These animals were used from time to time for testing the excretion of radioactive sodium and potassium. This entailed keeping the rats on a restricted regimen of Locke's solution with glucose for 3 days at a time. A temporary loss of body weight occurred with each experiment. The electrolyte excretion of these animals is reported in another communication.¹

Adrenalectomized rats given 1% NaCl *ad libitum* consume as much food as normal rats. The intake of food and of salt solution and the gain in body weight were measured on 2 groups of adrenalectomized rats and on 2 control groups for a period of 30 days. These data are shown in Chart II. The stock diet given these animals contained 1.6% sodium by analysis (=4% NaCl). The animals were kept in individual cages in a chamber kept at a constant temperature of 28.5°C. One group of 5 adrenalectomized rats was given tap water to drink. Their NaCl intake in the food averaged

¹ Anderson, E., Joseph, M., and Herring, V., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **44**, 482.

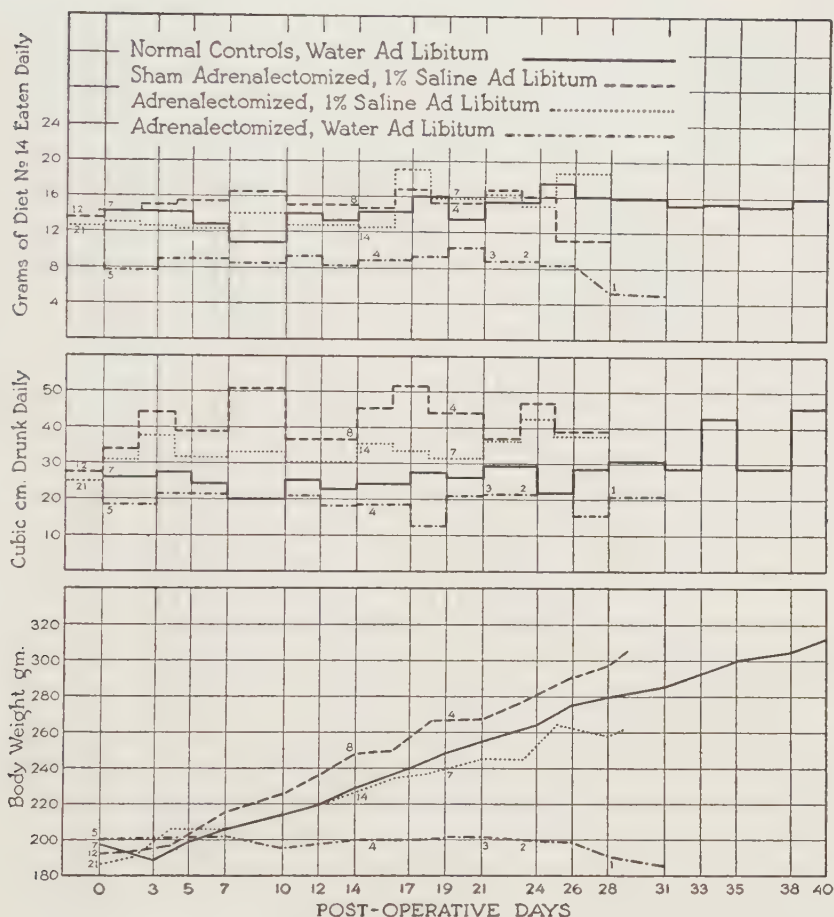


CHART II.

Showing the growth response and the food and fluid intake of adrenalectomized rats on 1% NaCl. Normal controls on tap water: total NaCl intake 601 mg daily. Sham adrenalectomized rats on 1% saline: total NaCl 1121 mg daily. Adrenalectomized rats on 1% saline; total NaCl 940 mg daily. Adrenalectomized rats on tap water: total NaCl 339 mg daily.

339 mg daily. Another group of 21 adrenalectomized rats was permitted to drink 1% NaCl *ad libitum*. Their total NaCl intake was 940 mg daily. One control group had been subjected to a sham adrenalectomy. This group was given 1% NaCl *ad libitum*. Their total NaCl intake was 1121 mg daily. The other control group consisted of normal rats which were given tap water to drink. Their NaCl intake was 601 mg daily. The adrenalectomized rats which had a total NaCl intake of 339 mg daily failed to grow and the animals died between the 14th and 31st days post-operative. The adrenalectomized rats with a higher NaCl intake, namely 940 mg, grew as

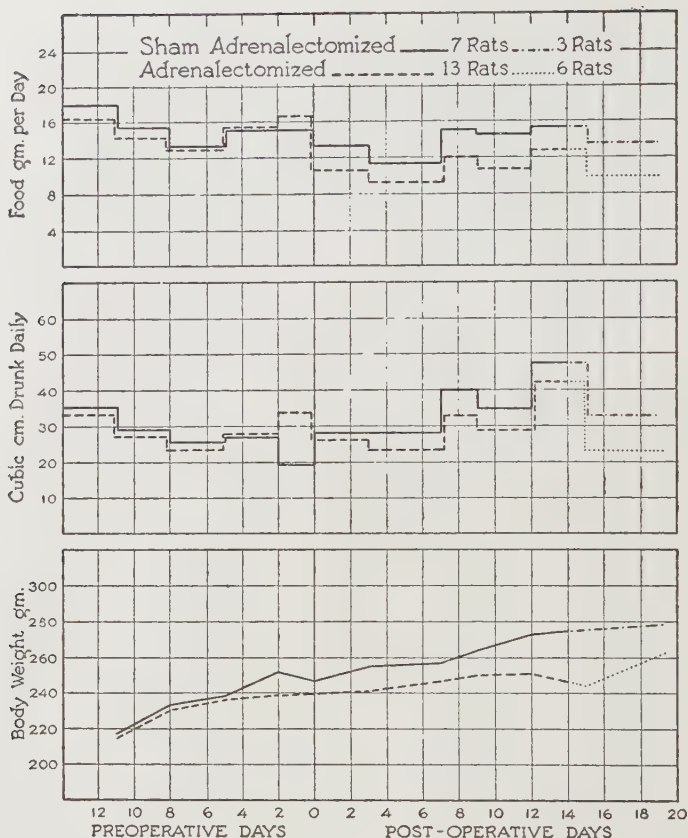


CHART III.

Showing the growth response and the food and water intake of adrenalectomized rats, given 16 cc of 5% NaCl daily. Sham adrenalectomized rats: total NaCl intake 1352 mg daily. Adrenalectomized rats: total NaCl intake 1234 mg daily.

well as normal animals. These animals were sacrificed on the 10th, 15th, 20th and 30th days post-adrenalectomy in order to determine the carbohydrate stores after feeding glucose. This is reported in another communication.²

In contrast to the beneficial effects of a 1% NaCl solution upon the growth and survival of adrenalectomized rats, it was found that larger amounts of NaCl in the drinking water proved to be injurious to such animals. This was tested out as follows: When adrenalectomized rats were given 4 cc of a 5% NaCl solution by stomach tube twice a day and allowed to drink tap water *ad libitum* and offered

² Anderson, E., Herring, V., and Joseph, M., PROC. SOC. EXP. BIOL. AND MED., 1940, to be published.

a standard diet which contained .7% sodium by analysis (=1.75% NaCl), they grew and remained in good health for an indefinite period. They resembled the adrenalectomized rats reported above which were kept on 1% NaCl. The total NaCl intake was practically the same for both groups, averaging about 650 mg. Another group of adrenalectomized rats was given 4 cc of 5% NaCl four times a day and allowed to drink tap water *ad libitum*. Their NaCl intake amounted to more than one gram per day. These animals resembled untreated adrenalectomized rats; they failed to grow and their average survival period was 19.5 days (with a range of 5-28 days). When 4 cc of 5% NaCl was given 6 times a day to adrenalectomized rats, the animals were all dead within 2 days. The urinary excretion of sodium and potassium in this group of animals is reported separately.¹

The higher amounts of NaCl decreased the food intake slightly for both adrenalectomized and control rats. Food records and water intake were kept on a group of 13 adrenalectomized rats and 7 controls which were given 4 cc of 5% NaCl 4 times a day. The NaCl intake for the adrenalectomized rats was 1234 mg daily, for the controls 1352 mg (Chart III.). The difference in food intake in these two groups was slight. There was no significant growth in either group. The animals were sacrificed on the 15th and 20th days post-adrenalectomy, in order to determine the ability of these animals to store carbohydrate. These data are reported separately.²

Summary. A daily intake of 650 to 940 mg NaCl for an adult adrenalectomized rat seems to be "optimal" in maintaining growth and apparent health. A daily intake of 339 mg is not sufficient for growth or survival. Large amounts of NaCl such as 1200 mg a day are injurious.

Salt After Adrenalectomy. II. Urinary Excretion of Radioactive Na and K in Adrenalectomized Rats Given Various Levels of Salt.*

EVELYN ANDERSON, MICHAEL JOSEPH AND VIRGIL HERRING.
(Introduced by Herbert M. Evans.)

From the Institute of Experimental Biology and the Department of Medicine of the University of California, Berkeley and San Francisco, California.

In an earlier communication¹ we reported the use of radioactive sodium and potassium in the detection of changes in the urinary excretion rate of sodium and potassium after adrenalectomy. It was found that adrenalectomized rats fed one of our stock diets which contained 1.75% NaCl, and given tap water to drink showed an increased rate of excretion of administered radioactive sodium and a diminished rate of excretion of radioactive potassium. The rate of excretion of these tagged electrolytes could be correlated with the excretion of body sodium and potassium. We also showed that the giving of a one percent sodium chloride solution to adrenalectomized rats instead of tap water corrected the wastage of sodium and the retention of potassium, so that these animals excreted these electrolytes in the same proportions as normal animals.

In the preceding communication² the growth and survival of adrenalectomized rats given 1% NaCl solution has been described. Out of a group of 25 adrenalectomized rats which were given 1% NaCl to drink and which were fed a diet which contained 1.75% NaCl, 10 lived beyond the 110th day after operation. The capacity of these animals to excrete given amounts of radioactive sodium and potassium was measured from time to time.

The methods used and the standardization of the conditions necessary for this study have been described previously.¹ The data of this study are given in Table I. It will be seen that adrenalectomized rats on 1% NaCl (with a total NaCl intake of 725 mg daily) at first excrete the administered radioactive sodium like the control animals but later show sodium retention. This discrepancy

* We wish to acknowledge the assistance of the Federal Works Progress Administration, Project No. OP 65-1-08-62, Unit A-5, and the Christine Breon Fund. We wish to thank Dr. John H. Lawrence, of the Radiation Laboratory of this University, for supplying us with the radioactive sodium and potassium.

¹ Anderson, E., and Joseph M., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **40**, 347.

² Anderson, E., Joseph, M., and Herring, V., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **44**, 477.

TABLE I.
Urinary Excretion of Na and K in Adrenalectomized Rats Given 1% NaCl.

Days post-adrenalectomy	Na ²⁴			Total Na			K ⁴²			Total K 90th	
	13th	26th	80th	126th	80th	5th	16th	48th	90th		136th
Group I											
Normal rats on 1% NaCl	36.7(5) * (33.0-40.0)†	35.0(10) (30.8-42.1)	31.9(3) (26.8-35.8)	31.9(3) (26.8-35.8)	81(3) (77-87)	10(5) (8.8-11.6)	9.7(5) (8.4-10.4)	9.0(10) (8.2-10.4)	11.9(3) (10.2-12.8)	11.9(3) (10.2-12.8)	43(3) (40-48)
Group II											
Adrenalectomized rats on 1% NaCl	35.7(5) (33.0-37.5)	32.3(6) (28.6-40.0)	24.6(2) (23.9-25.2)	17.8(5) (15.1-21.5)	70(2) (63-68)	9.6(5) (7.8-10.0)	9.0(5) (8.4-10.0)	10.8(6) (8.2-12.1)	12.3(5) (9.3-15.5)	12.6(5) (10.1-14.3)	46(5) (37-52)

*Number of animals in parentheses.

†Range of values.

increases as the period after adrenalectomy increases. This is in marked contrast to the behavior of untreated adrenalectomized animals, in which there is an increased excretion of sodium. The excretion of radioactive potassium in the adrenalectomized animals of this group was essentially the same as that of the control animals; as stated above the untreated adrenalectomized animal excretes a diminished amount of potassium. It can be noted that the excretion of body sodium and potassium bears a definite correlation to the excretion of radioactive sodium and potassium.

Increasing the amount of NaCl administered to adrenalectomized rats beyond an optimal level, proved to be injurious as described in the preceding communication.² However, these high doses of NaCl enabled the adrenalectomized rat to continue to excrete radioactive sodium and potassium like normal controls. These data are given in Table II. The animals in Group I of Table II received

TABLE II.
Urinary Excretion of Na and K in Adrenalectomized Rats on High NaCl Intake.

Days post-adrenalectomy	Sodium		Potassium K ⁴²		Total K 24th mg
	Na ²⁴ 20th %	Total Na 20th mg	12th %	24th %	
Group I (4 cc 5% NaCl 2x daily = 400 mg)					
Adrenalectomized rats	36.2(5)* (33.0-40.0)†	—	8.6(5) (7.8-8.8)	9.2(5) (8.4-10.4)	—
Normal rats	35.0(10) (30.8-42.1)	—	9.7(5) (8.4-11.5)	—	—
Group II (4 cc 5% NaCl 4x daily = 800 mg)					
Adrenalectomized rats	31.6(5) (27.2-37.5)	89(5) (85-94)	8.9(5) (8.8-10.0)	10.0(3) (9.5-10.4)	39(3) (36-43)
Normal rats	36.0(3) (33.0-37.5)	83(3) (79-88)	7.7(3) (6.4-8.8)	9.4(3) (9.1-9.5)	40(3) (36-43)

*Animals per group in parentheses.

†Range of values.

400 mg NaCl administered in a 5% NaCl solution, in addition to about 245 mg of NaCl in the food. As described previously,² these animals continued to grow and remained in a healthy condition. Those of Group II received 800 mg of NaCl in addition to approximately 245 mg of NaCl in the food. The adrenalectomized animals of this group were all dead by the 28th post-operative day. However, in both groups of adrenalectomized rats the excretion of electrolytes resembled that of normal rats. The total sodium and potassium excreted showed a definite correlation with the percent of radioactive sodium and potassium excreted.

Summary. The administration of sodium chloride to adult adrenalectomized rats in amounts varying from 650 mg to 1 g daily prevents the urinary sodium wastage and potassium retention which characterizes adrenalectomized animals.

11502 P

Oxidation of Tyrosine by Ultraviolet Light in its Relation to Human Pigmentation.

STEPHEN ROTHMAN. (Introduced by G. F. Dick.)

From the Department of Medicine, Section of Dermatology, and the Department of Pharmacology, University of Chicago.

In the skin of mammals tyrosinase never has been conclusively demonstrated. It has been assumed¹ that the immediate precursor of melanin in mammalian skin is 3-4 dihydroxy-phenylalanin ("dopa") which becomes oxidized to melanin by an intracellular specific oxidase present only in normal functioning melanoblasts. The question has remained unsettled from where this dopa may originate; whether it is formed from tyrosine in the blood² or in the skin.

Arnow³ demonstrated the formation of dopa by exposure of tyrosine solutions to ultraviolet radiation. As shown in our laboratory, this process needs a strikingly long irradiation time, namely 8-30 times as much as necessary for slightest pigmentation of human skin. In the presence of ferrous salts, however, the formation of dopa from tyrosine by ultraviolet irradiation is accelerated to such a degree that it may serve as a model of the biologic formation of dopa in human skin.

Samples containing mixtures of tyrosine and ferrous salts, irradiated with 1-3 "threshold erythema doses" yield measurable amounts of dopa but no melanin. When such irradiated samples are kept in the dark, progressively increasing amounts of precipitated melanin are formed after 16-24 hours. In this way the latent period of pigment formation in human skin is simulated by the *in vitro* experiments.

The late formation of melanin occurs for the greatest part at the

¹ Bloch, Br., *Jadassohn's Handb. d. Haut. u. Geschlkr.*, 1927, **1**, 434.

² Rothman, S., *Z. f. d. ges. exp. Med.*, 1923, **36**, 398.

³ Arnow, L. E., *J. Biol. Chem.*, 1937, **120**, 151.

expense of dopa which has been formed during the radiation. However, in a few instances a slight decrease of tyrosine and a transitory increase of the dopa concentration could be observed within 1-2 hours after irradiation. This "after effect" of irradiation may be due to formation of rather stable peroxides by ultraviolet rays.

In tyrosine-ferrous salt mixtures the dopa concentration increases with continued irradiation to a certain maximum. If 50 mg % tyrosine solutions are irradiated this maximum amounts to about 5 mg %. Later, in spite of continued irradiation, the dopa concentration remains unchanged because dopa formation and oxidation of dopa to melanin keep balance with each other. Or, the dopa concentration decreases because more melanin than dopa is formed.

In long-lasting irradiation experiments it was found that in spite of a steady decrease of the tyrosine concentration the amount of melanin produced does not exceed a certain maximum. This is due to a decomposition of melanin into lighter colored, soluble products by the continued irradiation.

For clinical actinotherapy this finding indicates that after maximal pigmentation has been reached, continued irradiation does not produce a static but a dynamic equilibrium in which pigment formation and decomposition are kept in balance. Some clinicians have claimed that ultraviolet light treatment should be administered intermittently in order to assure an optimum therapeutic effect by "depigmentation periods." The experimental results, however, seem to obviate the necessity of such a procedure, and it appears justified to continue sunshine treatment in patients who already are tanned to a maximum, because biologic action of the rays continues.

Ultraviolet radiation acts on tyrosine in presence of ferrous salts similarly to tyrosinase in every detail. This fact supports the assumption that in mammals too, in which tyrosinase is not present, the primary precursor of melanin is tyrosine. The formation of dopa may occur in the skin by the action of ultraviolet rays if non-specific oxidation catalysts are present.

Summary. The oxidation of tyrosine to melanin by exposure to ultraviolet light in presence of ferrous salts serves as a model of pigment formation by sunshine in human skin. Continued irradiation of tyrosine-ferrous salt mixtures leads to a dynamic equilibrium in which formation and decomposition of melanin are kept in balance.

11503 P

Free and Combined Sulfanilamide in Material Drained from the Human Biliary Tract.

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The concentrations of free and of combined (presumably acetyl) sulfanilamide have been determined in the material collected through T-tubes inserted in the bile ducts of human subjects after operations upon the biliary system. Bile was clarified by a mixture of trichloroacetic and phosphotungstic acids. Free sulfanilamide was determined by the method of Marshall and Litchfield¹ after the addition of acetone to the filtrate from the pigments etc. removed by this procedure. In the determination of combined sulfanilamide, the filtrate was treated with hydrochloric acid and hydrolyzed for an hour in a boiling water bath before the diazo reaction was carried out. Both free and acetyl sulfanilamide* were added to the bile and were recovered quantitatively by this procedure, but the method of precipitating could not be considered as wholly satisfactory, for a trace of pigment (apparently biliverdin) was present after treatment in a fair number of specimens from some of the patients studied. The method of Marshall and Litchfield was also used in the study of urine specimens collected simultaneously with the bile from a number of the patients. Blood analyses were carried out by the technique of Bratton and Marshall.²

Although the clarification of some of the bile specimens was not satisfactory the results of 10 studies upon 5 patients agreed closely together, and justify the following statements. There was no compound in the clarified bile which gave a reaction with the diazo technique used. Shortly after one to 2 g of sulfanilamide were given by mouth, the free drug appeared in the bile draining from the T-tube, but regularly in a concentration lower than that present in the blood. This finding differs from results previously reported upon human bile from the gall bladder³ for the concentration of free sulfanilamide in such material is frequently higher than it is

¹ Marshall, E. K., Jr., and Litchfield, J. T., *Science*, 1938, **88**, 85.

* The acetyl sulfanilamide used was a synthetic product furnished through the courtesy of the National Aniline and Chemical Company.

² Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

³ Bettman, R., and Spier, E., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 463.

TABLE I.
Concentration of Sulfanilamide in Urine, Blood and Drainage from the Biliary Tract.

Period 4 hr each	Fluid drained from biliary tract						Blood		Urine	
	Apr. 6		Apr. 11		Apr. 14		Apr. 11 Apr. 14		Apr. 14	
Concentration of sulfanilamide—mg per 100 cc.										
	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total
Before	—	—	0.00	0.00	—	—			—	—
	2 g (xxx grains) of sulfanilamide given by mouth.									
1st after	0.76	0.70	1.60	1.66	1.77	1.75	2.87	3.70*	14.3	23.6
							2.25	4.70†		
2nd "	1.35	1.77	1.72	2.11	0.40	0.42‡			30.8	45.2
3rd "	1.46	1.68	—	—	1.45	1.84			27.6	29.7
	2 g (xxx grains) of sulfanilamide given by mouth.									
4th "	—	—	—	—	1.58	1.74	1.80	2.50*	20.8	37.7
5th "	—	—	—	—	1.92	2.54			19.0	29.9
6th "	—	—	—	—	2.37	2.96			18.1	22.3
7th "	—	—	—	—	2.56	3.20			26.6	32.3

*Blood collected April 11, 1940.

†Blood collected April 14, 1940.

‡About 5 cc of very thin bile drained from the tube during this 4-hour period.

The time of the 4-hour periods of collection (before, 1st after, etc.) is calculated from time when the first dose of sulfanilamide was administered.

The blood specimens were collected at the mid-points of the 4-hour periods against which they are recorded.

in the blood. Later, the free sulfanilamide in our experiments approximated more nearly the concentration in the blood, as would be expected from the water content of the two fluids⁴ but the relationship was not directly proportioned to the water content. Sulfanilamide could be demonstrated in the bile 24 hours after 2 g had been taken by mouth.

During the first 4 hours after the administration of such single doses as were used in these experiments, no demonstrable amount of combined form of the drug could be demonstrated in the bile, although both free and combined forms were found in the blood during this period. After 4 hours, both compounds were present, although the concentrations of the combined forms were small, for only 10 to 20% of the total sulfanilamide occurred as the combined derivative at periods when analyses of blood and urine showed that approximately half of the total sulfanilamide reacted with the diazo reagents only after acid hydrolysis. If acetyl sulfanilamide is formed in the liver, as current experimental work indicates⁵ it is apparently not discharged in large amounts directly into the bile.

⁴ Marshall, E. K., and Cutting, W. C., *J. Am. Med. Assn.*, 1937, **108**, 953; *J. Pharm. Exp. Therap.*, 1937, **61**, 196.

⁵ Harris, J. S., and Klein, J. R., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 781; Klein, J. R., and Harris, J. S., *J. Biol. Chem.*, 1938, **124**, 613.

The findings described above, except the presence in the bile for 24 hours of appreciable amounts of free and combined sulfanilamide, are illustrated in the results of these experiments upon one subject shown in the table. As already stated, these results are essentially similar to studies made upon 4 other subjects. The experiment presented also shows that when a second dose of sulfanilamide was ingested at a time when the drainage from the biliary tract contained the drug in both the free and the combined forms, the increase in the amount of the total compound was largely or wholly in the free state. In the urine on the other hand, the larger proportion of the increase was the combined (acetyl) derivative.

11504

Occurrence of Precipitation Zones in Mixtures of Serum and Sodium Desoxycholate; Significance in Pneumococcolysis.*

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Observations by Ransom,¹ Bayer,² Sellards,³ Ponder,⁴ and Williams⁵ have shown that the lysis of red cells by bile salts is inhibited in the presence of serum. Wieland,⁶ and Donnelly and Mitchell⁷ have subjected this fixation of bile salts by serum to quantitative study. The latter felt that the bile salt-serum reaction, which manifested the Danysz effect, was one of adsorption rather

* This study received additional financial support from the Metropolitan Life Insurance Company, and from Mr. Bernard M. Baruch, Mr. Bernard M. Baruch, Jr., Miss Belle N. Baruch, and Mrs. H. Robert Samstag.

[†] Littauer Fellow in Pneumonia Research.

[‡] Dazian Fellow.

1 Ransom, F., *Deutsche Med. Wochenschr.*, 1901, **27**, 194.

2 Bayer, G., (a) *Biochem. Z.*, 1907, **5**, 368; (b) *idem.*, 1908, **9**, 58.

3 Sellards, A. W., *J. H. Hosp. Bull.*, 1908, **19**, 268.

4 Ponder, E., *Proc. Roy. Soc., B.*, 1922, **93**, 86.

5 Williams, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 916, 918.

6 Wieland, H., *Naunyn-Schmiedeberg, Arch. Pharm. und exp. Path.*, 1920, **86**, 79, 92.

7 Donnelly, J. L., and Mitchell, A. G., *Am. J. Physiol.*, 1927, **79**, 297.

TABLE I.
Lysis of an 18-hour Culture of *Pneumococcus* III.
1 ml of 4% serum broth culture plus 1 ml of various concentrations of sodium desoxycholate, in plain broth.
Final Dilution of Sodium Desoxycholate.

37°C	1:250	1:500	1:750	1:1,000	1:1,250	1:1,500	1:2,500	1:5,000	1:10,000	Broth Control
Serum	Immed.	T+	T+	T++	T++	T++	T++	T++	T++	+
Broth	30'	C	T+	T+	T+	T+	T+	T++	T++	+
Culture	60'	C	T+++	T+++	T++	C	T++	T++	T++	+

Key to all tables:

C = Clear.

T+, T++, T+++ = Various degrees of turbidity.

ff = Fine flocculation.

cf = Coarser flocculation.

Immed. = Immediately.

OV = Over night.

than of chemical combination. They demonstrated that the reaction quantitatively followed the adsorption equation, $\frac{X_n}{C} = K$. This, however, according to Sobotka,⁸ is not appreciably different from the earlier mass action formulation of Wieland, $\frac{aB}{(A-a)^2} = K$. Carlinfante,⁹ has recently reported the occurrence of turbidity in mixtures of 5% human serum and diluted ox bile. In his experience, turbidity did not occur with dilutions of bile greater than 1 to 40.

During the course of experiments dealing with the quantitative determination of pneumococcal capsular polysaccharide in lysates, we noted certain irregularities in the clearing of 4% serum broth cultures (Pn. III) by solutions of sodium desoxycholate. Portions of this culture had been diluted 1 to 2 with broth containing the desired concentration of sodium desoxycholate, so that the final mixture contained horse serum in a 2% concentration. Under these conditions cultures containing concentrations of desoxycholate ranging between approximately 1 and 2 mg per ml failed to clear after several hours at 37° C and overnight refrigeration while with several higher and lower concentrations the same cultures cleared completely in one half hour at 37° C. (Table I). Smears and cultures from the turbid fluids containing 1 to 2 mg per ml of desoxycholate, however, failed to reveal bacteria.

The reactions were, therefore, repeated with plain as well as serum broth cultures. The middle zone of turbidity did not develop with plain broth cultures nor did control tubes of plain broth, without organisms, become turbid in the presence of those concentrations of desoxycholate employed in the lysis studies. The pneumococci grown in serum-broth were washed 3 times in saline and one-half the sediment resuspended in plain saline and the other half in 4% serum saline. These were also diluted 1 to 2 with broth containing various concentrations of sodium desoxycholate. The plain saline suspension cleared completely with concentrations of sodium desoxycholate up to and including 1-2500; the serum saline suspension became turbid in the same zone encountered with the unwashed serum broth cultures. Control tubes with a final concentration of 2% serum became turbid in the identical zone. (Table II).

We employed the technique of Donnelly and Mitchell,⁷ mixing varying concentrations of sodium desoxycholate with 2% horse

⁸ Sobotka, H., *Physiological Chemistry of the Bile*, Williams and Wilkins, Baltimore, 1937, page 131.

⁹ Carlinfante, E., *Sperimentale, Arch. di Biol.*, 1938, **92**, 8.

TABLE II.
Lysis of 18-hour Plain Broth Culture of *Pneumococcus* III and of Washed Serum Broth Culture Resuspended in Saline with or without Serum.
(Final concentration of serum—2%.)
Final Dilution of Sodium Desoxycholate.

37°C		1:250	1:500	1:750	1:1,000	1:1,250	1:1,500	1:2,500	1:5,000	1:10,000	Diluent Control
Plain broth*	Immed. 30' OV	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C
Washed sediment suspension in saline	Immed. 30' OV	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C
Washed sediment suspension in 4% serum saline	Immed. 30' OV	C C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C
4% serum broth without organisms	Immed. 30' OV	C C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C	T+ C C

*The density of this culture was adjusted to equal that of the 18-hour culture described in Table I (determined by photorefractometer).

TABLE III.
Development of Turbidity and Flocculation in Mixtures of Sodium Desoxycholate and Normal Horse Serum. Final Concentration of Horse Serum 2% in All Tubes.
Final Concentration Sodium Desoxycholate.
Mg per ml.

At room temperature	4.0 (1:250)	3.4	3.0	2.4	2.0 (1:500)	1.6	1.2	1.0 (1:1000)	Controls		
									0 (2% serum)	0.6 (2% serum)	1.6 (No serum)
5'	C			T+	T+	T+	C	C	C	C	C
10'	C	C	C	T+	T+	T+	C	C	C	C	C
15'	C	C	C	T+	T+	T+	C	C	C	C	C
30'	C	C	C	T+	T+	T+	T+	C	C	C	C
60'	C	C	C	T+	T+	T+	T+	C	C	C	C
90'	C	C	C	T+	T+	T+	T+	C	C	C	C
120'	C	C	C	T+	T+	T+	T+	C	C	C	C
OV	C	C	C	T+	T+	T+	T+	C	C	C	C
				T+	ff	cf	ff				

Powdered sodium desoxycholate, C.P., was dissolved directly in plain broth for use in experiments recorded in Tables I and II. The salt was evaporated from a 1% alcoholic solution before use in the experiment of Table III.

serum broth to a constant volume of 5 ml.[§] Table III shows the development of turbidity and flocculation in these mixtures and in the control tubes. Turbid fluids were not cleared by centrifugation at 2,000 rpm for 20 minutes nor by boiling. Addition of 3% acetic acid precipitated the bile salt. Normal NaOH in excess cleared the turbid mixture.

It will be noted in Table III that a 2% horse serum broth remained clear in the presence of 4 mg per ml of sodium desoxycholate while a tube containing 2 mg per ml became turbid promptly and had developed fine flocculation after standing overnight. To 5 ml of the turbid solution containing 2 mg per ml of the bile salt an additional 10 mg of dry sodium desoxycholate was added. There was prompt and complete clearing of the solution. The addition of an excess of serum to another turbid fluid failed to clear the mixture. The pH of the solutions containing 2 and 4 mg per ml of sodium desoxycholate, respectively, was determined with the quinhydrone electrode and both were found to be at 7.3. An alteration of pH was apparently not the factor responsible for the clearing.

We have also observed that solutions of sodium desoxycholate of greater than 2.0 mg per ml concentration, either in broth or in phosphate buffer near neutrality, gelled after standing at room temperature. This gelation occurs in the absence of serum and, as was also noted by Schaub and Reid,¹⁰ disappears on warming. Preliminary observations apparently indicate that gelation is more rapid and complete at neutrality than it is on the alkaline side of neutrality. The occurrence of this gel in cultures containing bile salts at a pH of 6.8 has been previously described by Mair¹¹ and is an evidence of their colloidal character of which others have been described.^{4, 12-14}

The present observations indicate that serum interferes with the interpretation of lysis in the presence of certain concentrations of sodium desoxycholate. However, several text-books offer con-

§ The reactions were unaffected when a Sørensen phosphate buffer at a pH of 7.3 was substituted for peptone broth as diluent. Stock powdered sodium desoxycholate did not dissolve in broth until heated to 65°C. After evaporation from alcohol, however, rapid solution took place at room temperature. The pH of the mixture of serum with the highest concentration of desoxycholate employed was 7.3, determined with the quinhydrone electrode.

¹⁰ Schaub, I. G., and Reid, R. D., *J. A. M. A.*, 1938, **111**, 1285.

¹¹ Mair, W., in *A System of Bacteriology*, Med. Res. Council, 1929, London, Vol. II, page 168.

¹² Achard, C., Boutarie, A., and Berthier, P., *Compt. Rend. Acad. Sci. (Paris)*, 1937, **204**, 1049.

¹³ Bashour, J. T., and Bauman, L., *J. Biol. Chem.*, 1937, **121**, 1.

¹⁴ Roepke, R. R., and Mason, H. L., *J. Biol. Chem.*, 1940, **133**, 103.

flicting opinions regarding the influence of serum on the lysis of pneumococci by bile salts.^{11, 15, 16}

Summary. Turbidity and flocculation developed in 2% horse serum broth containing concentrations of sodium desoxycholate between 1.2 and 2.4 mg per ml at a pH of 7.3 and cleared on the addition of an excess of sodium desoxycholate. Apparent failures of lysis of serum broth cultures of pneumococci were due to the occurrence of such turbidity because no bacteria were found in these mixtures.

11505

Influence of Arsenicals, Bismuth and Iron on the Plasma Ascorbic Acid Level.

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Several reports indicate that the poisonous effects of a number of drugs like benzene, lead,¹ phenylcinchoninic acid,² and glycerol³ and especially the arsenicals¹ may be counteracted successfully by giving suitable doses of ascorbic acid.

From examination of the urine Dainow⁴ concluded that patients who showed symptoms of intolerance to arsenicals were in a state of hypovitaminosis C. By administration of ascorbic acid, these hypersensitive patients became able to tolerate neoarsphenamine. Other investigators⁵⁻¹¹ reporting similar observations, emphasize

¹⁵ Boecker, E., and Kauffmann, F., *Bakteriologische Diagnostik*, 1st Ed., 1931, Berlin, J. Springer, p. 71.

¹⁶ Park, W. H., and Williams, A., *Pathogenic Microorganisms*, 10th Ed., 1933, Lea and Febiger, Phila., p. 353.

¹ Abt, A. F., and Farmer, Chester J., *The Vitamins*, Chapter XXII, American Medical Association, Chicago, 1939.

² Bertellotti, L., *Minerva, Medic.*, 1939, **30**, 254.

³ Pfeiffer, C., and Arnoe, T., *Proc. Soc. Exp. Biol. and Med.*, 1937, **37**, 467.

⁴ Dainow, T., *Presse méd.*, 1937, **45**, 1670; *Annal. Dermat. et Syphil.*, 1939, **10**, 139.

⁵ Landfisch, S., *Polska gas. lek.*, 1937, **16**, 575; *J. A. M. A.*, 1937, **109**, 834.

⁶ Cormia, F. E., *Canad. Med. Assn. J.*, 1937, **36**, 392.

⁷ Montesano quoted by Bertellotti.²

⁸ Biss quoted by Dainow.⁴

⁹ Tibor, S., *Orvosi Hetilap*, 1939, **83**, 811.

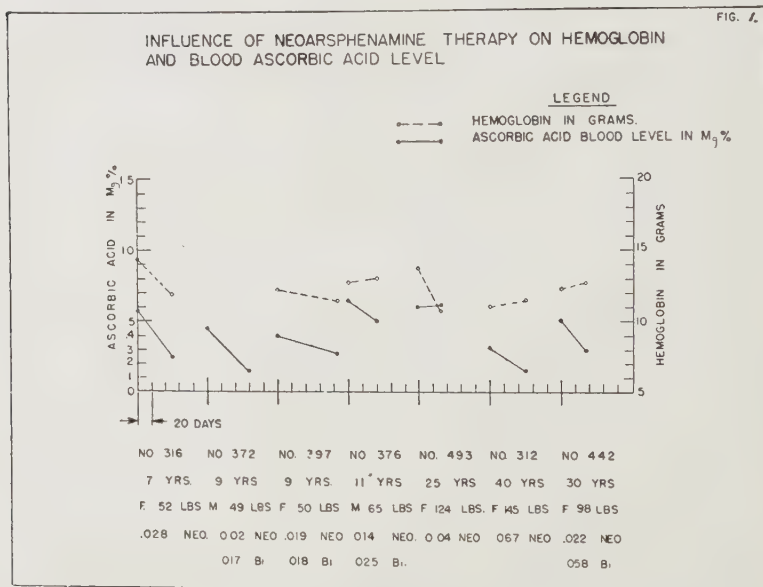
¹⁰ Santiago, A., *Zbl. Haut und Geschl. Krkh.*, 1938, **60**, 74.

¹¹ Takahashi quoted by Bertellotti.²

the fact that in certain hypersensitive cases ascorbic acid gave favorable results after other methods of detoxification such as the administration of glucose, invert sugar, and calcium or sodium thiosulfate had failed.

After a suitable method for determining plasma ascorbic acid had been developed, studies were commenced in 1938 on syphilitic patients showing symptoms of intolerance to arsenicals. A more extended systematic study of this problem was recently made possible in connection with our Nutritional Survey of the syphilitic patients attending the Municipal Social Hygiene Clinic, Chicago.*

It was noted early in the work that patients hypersensitive to neoarsphenamine in whom treatment had to be discontinued because of severe reactions† required exceedingly large oral doses of ascorbic acid‡ to bring their plasma levels up to optimal values (1.0 mg %



These data demonstrate that neoarsphenamine (in contrast to bismuth) exerts a depressive action on the plasma ascorbic acid level. In 6 out of the 7 cases a distinct decrease in the plasma level occurs. The hemoglobin is but slightly affected.

* We are indebted to Dr. O. C. Wenger, Senior Surgeon, U. S. Public Health Service, and to Dr. Herman N. Bundesen, President of the Chicago Board of Health, for the facilities and opportunity of studying patients attending the Municipal Social Hygiene Clinic. We also wish to acknowledge the cooperative assistance of Dr. G. G. Taylor, Director of the Clinic.

† These reactions consisted of nausea, vomiting, fever, dermatitis, and in one case hepatitis.

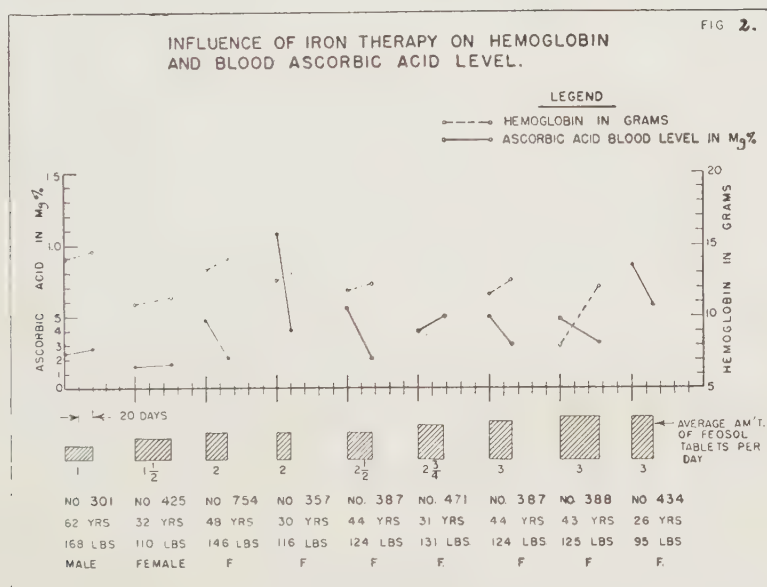
‡ We are indebted to Merck and Company, Inc., Rahway, N. J., for a generous supply of Cebione (ascorbic acid) used in this investigation.

or above). When showing severe symptoms of intolerance, a decline of the plasma level occurred in spite of the oral administration of ascorbic acid during treatment. It was frequently observed that a marked lowering of the plasma level followed the administration of neoarsphenamine in patients showing no intolerance to the drug (Fig. 1).

When bismuth was given in doses routinely used for antiluetic treatment, no appreciable effect was observed either on the plasma ascorbic acid or hemoglobin levels.

Striking effects were observed on the administration of ferrous sulphate (Feosol[§]). Doses smaller than 6 grains daily were ineffective. However, 6 grains or more caused a sharp drop in the plasma ascorbic acid level as will be seen from Fig. 2.

Exactly the same type of response was observed when ferrous sulphate was given to 12 patients receiving bismuth therapy. The greatest drop in plasma ascorbic acid level occurred in patients taking

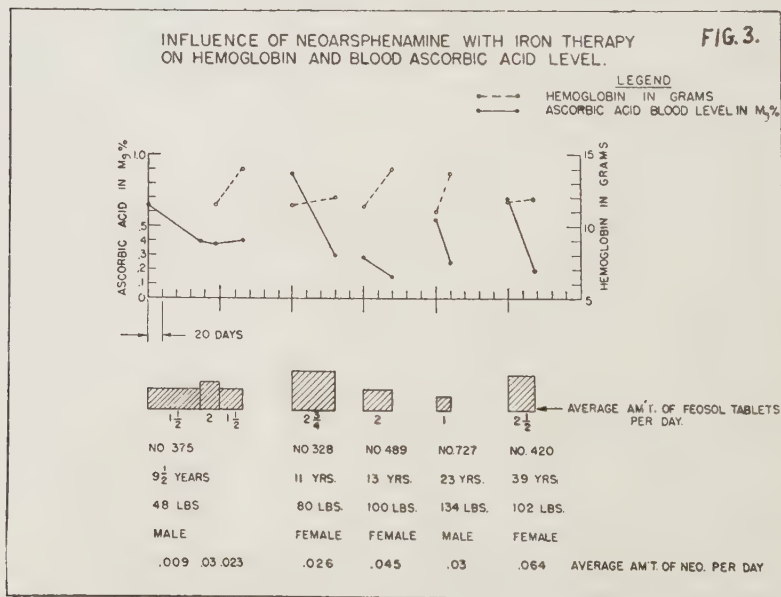


Patients who were at a rest period and did not receive antiluetic treatment were given iron orally in the form of ferrous sulphate ("Feosol" tablets, each containing 3 grains of ferrous sulphate). The hemoglobin rose in every case examined. However, when the dosage of "Feosol" amounted to 2 or more tablets per day there was a sharp drop in the plasma ascorbic acid level in 6 out of the 7 patients.

§ We are indebted to Smith, Klein and French Laboratories, Philadelphia, for a liberal supply of Feosol Tablets. Each tablet contains 3 grains of ferrous sulphate. The other ingredients are of no significance here.

ferrous sulphate while receiving neoarsphenamine. These data are presented in Fig. 3. It should be emphasized that in spite of this decline in plasma ascorbic acid level the hemoglobin rose in practically every case. This observation is in good accord with the findings of Moore, Bierman and Minnich,¹² who noted a definite decrease in plasma ascorbic acid following a rise in serum iron and hemoglobin after ingestion of large amounts of ferrous or ferric salts.

Our observations suggest 3 types of action of drugs containing heavy metals on plasma ascorbic acid. Bismuth is without influence. Iron causes a marked decrease, which may be of significance in rapid hemoglobin formation. Arsenic in drugs as neoarsphenamine, lowers plasma ascorbic acid, which in some cases may be an attempt on the part of the organism to detoxify the drug. As evidence of detoxification, patients previously hypersensitive to arsenicals have been permitted to resume treatment upon administration of suitable doses of ascorbic acid, when the optimal plasma level was attained. Repeated plasma analyses must be made to determine the amount



These data present the simultaneous action of neoarsphenamine and Feosol on plasma ascorbic acid level and hemoglobin. It will be seen that plasma ascorbic acid values dropped from fairly normal to decidedly low levels. The hemoglobin, on the other hand, rose more or less in every case.

¹² Moore, C. V., Bierman, H., and Minnich, V., Centr. Soc. Clinie. Res., 12th Ann. Meet., 1939.

of ascorbic acid required for maintenance of the optimal plasma level during treatment. In conclusion our data indicate the necessity for a high ascorbic acid intake during certain types of medication with heavy metal compounds, to meet excessive requirements either for physiological demands, or for detoxification of drugs in certain cases before therapeutic levels can be attained.

We wish to acknowledge our indebtedness to H. J. Fagen and J. Meyer for much of the analytical data reported here.

11506 P

Respiratory Metabolism of Pigeons after Adrenalectomy and its Increase by Prolactin.

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Recent experience in the maintenance of adrenalectomized animals in fair or good condition without use of cortical hormones gives new interest and value to measurement of the basal metabolism of such animals, and the adrenal-pituitary relationship is now the subject of much investigation. The pigeon has been found useful in such studies. It seems to survive complete adrenalectomy readily, though it is best to do the operation in two stages and to inject desoxycorticosterone just before the second operation; thereafter pigeons maintain themselves well without special nutritional or hormonal supplements. Repeated metabolism measurements have been made on 12 such pigeons of various races (both sexes) and on 4 of these birds the ability of prolactin to increase the B.M.R. was demonstrated.

Thirty measurements made at 30°C indicate that adrenal removal in pigeons has little effect on heat production; a decrease of 6% was found. Measured at 25°C this decrease was also 6%. Respiratory quotients obtained after a 24-hour fast were the same in operated (0.73) and intact pigeons. In 10 tests made on birds from which a single adrenal was removed no significant effect was observed. The effect of adrenalectomy on the metabolism of the bird is thus found to be less though similar in direction to that previously reported by others for certain mammals. Interpretation

of depressions noted in most mammalian tests is usually obscured by the regimen imposed to insure survival or by poor condition of the animals. In pigeons it seems probable that the observed small decrease in B.M.R. was the indirect result of some (presumptive) reduction in bodily activity and of some protection against low temperature. One thyroidectomized pigeon showed a 10% decrease in metabolism following adrenalectomy. Body weights may be well maintained for at least a few months. Measurements were made at intervals varying from 2 days to 4 months after operation.

Four adrenalectomized pigeons with an average B.M.R. of 3.78 calories per kilo-hour were injected with 5 mg (20 units) prolactin daily for 3 days. The heat production of these 4 birds was then +7, +34, +51 and +56. The preparation used (495H2) had been heated to 60°C, at pH 8.0, for 5 hours. This treatment should have been particularly adverse to glycotropic and growth factors if they were present. The prolactin used gave no apparent increase in dove thyroids, and contained little FSH. One of the 4 pigeons used in this test was completely thyroidectomized, and had previously shown no increase in B.M.R. after 5 and 8 daily injections of 4 mg of a prolactin-free preparation of thyrotropic hormone. In this thyroidectomized-adrenalectomized pigeon prolactin increased the B.M.R. by 34%. Earlier studies^{1, 2} have shown that prolactin increases the B.M.R. of hypophysectomized and thyroidectomized pigeons when measured at 30°C. Contrary to an earlier assumption² the present tests indicate that this action of prolactin is not mediated in part by the adrenals.

¹ Riddle, O., Smith, G. C., Bates, R. W., Moran, C. S., and Lahr, E. L., *Endocrinol.*, 1936, **20**, 1.

² Riddle, O., Smith, G. C., and Dotti, L. B., *Am. J. Physiol.*, 1938, **123**, 171.

11507 P

Cyanosis Produced by Anastomosis of Pulmonary Artery to Left Auricle.

MILTON MENDLOWITZ AND ALAN LESLIE.

(Introduced by G. Shwartzman.)

From the Laboratories of the Mount Sinai Hospital, New York City.

Cyanosis, such as occurs in congenital heart disease, is caused by a shunt of unoxygenated blood from the right side of the heart to the left. In order to reproduce this experimentally, it is apparent that a communication must be made at a point where unoxygenated is at a higher pressure than oxygenated blood. The only surgically accessible site for this procedure is the point of contiguity of the main and left pulmonary artery on the one hand, and the pulmonary vein or the left auricular appendage on the other. It was found that because of its relatively small size, the pulmonary vein could not be used.

The procedure therefore employed is as follows: A large dog anesthetized with sodium pentobarbital is placed on its right side, artificial respiration being maintained by intermittent positive pressure insufflation via a trans-oral tracheal cannula. An incision is made in the fourth left intercostal space, the ribs retracted, and the lung packed posteriorly. The pericardium is then incised posterior and parallel to the phrenic nerve, thereby exposing the site for the anastomosis. After isolation of the left pulmonary artery by dissection, an especially designed U-shaped serrefine is placed longitudinally on the main and left pulmonary artery. Blood flow to the right lung is not impaired by this maneuver. A rubber covered short intestinal clamp is then applied to the base of the left auricular appendage. Parallel incisions are now made in the isolated portions of the artery and auricle, which are anastomosed with a vaselized arterial silk suture on an atraumatic needle. Approximation of intima to endocardium is accomplished by the use of an everting, continuous mattress suture. After the operation the animal is heparinized, according to the method of Murray, *et al.*¹

This procedure has been performed successfully in 3 dogs, in each of which pronounced cyanosis of the tongue and depression of the oxygen content of the arterial blood were observed. Long term observations on these animals, as well as further experiments are now in progress.

¹ Murray, D. W. G., Jaques, L. B., Perrett, T. S., and Best, C. H., *Surgery*, 1937, **2**, 163.

Diuretic Effect of Progesterone.*

HANS SELYE AND LUCY BASSETT.

From the Department of Anatomy, McGill University, Montreal, Canada.

In the course of various experiments in which progesterone had been administered to normal and hypophysectomized rats we observed that this compound greatly increases urine output. We felt that an action of the corpus luteum hormone on diuresis may perhaps throw some light on the cause of the changes in water metabolism observed during normal and pathological pregnancies. Therefore this effect has been subjected to a quantitative analysis in normal and hypophysectomized rats whose urine was collected daily in metabolism cages.

Twenty-four albino females with an average weight of 105 g have been divided into 4 groups of 6. Group I was hypophysectomized and then given daily subcutaneous injections of 10 mg of progesterone in 0.4 cc of peanut oil. Group II was hypophysectomized and given daily subcutaneous injections of 0.4 cc of peanut oil only. Groups III and IV remained intact but received the same injections as Groups I and II respectively. Since hypophysectomy in itself is usually accompanied by a transitory marked diuresis and since the animals need some time to become used to life in individual metabolism cages and daily injections, we did not collect the urine during the first fortnight. After this however, accurate measurements of water intake and output were made on every animal during the 6 subsequent days. Table I shows the urine output during one day. For the sake of brevity the values obtained on other days are omitted since they were essentially the same as those

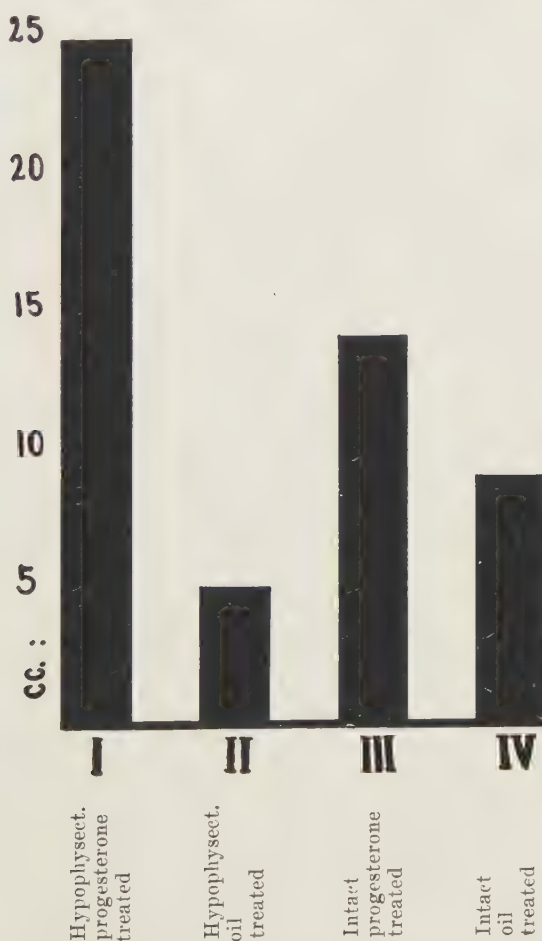
TABLE I.
24-hour Urine Excretion Under the Influence of Progesterone in Normal and Hypophysectomized Rats Expressed in cc.

Rat No.	1	2	3	4	5	6	Avg
I Hypophysectomized progesterone treated	11	46	12	21	25	22	23
II Hypophysectomized oil treated	0	4	2	5	2	10	4
III Intact progesterone treated	19	16	13	16	15	10	15
IV Intact oil treated	6	11	8	9	9	6	8

* The expenses of this investigation were defrayed by a grant received from the Schering Corporation of Bloomfield, N. J., through the courtesy of Dr. G. Stragnell. The progesterone was kindly supplied by Dr. E. Schwenk of the same Corporation.

given in the Table. A summary of the average daily output throughout the 6-day observation period is given in Graph I.

We believe that our experiment clearly indicates that progesterone increases the urine output both in intact and in hypophysectomized rats. Although the hypophysectomized controls excreted only about half as much urine as the normals, the progesterone-treated hypophysectomized animals voided much more than any other group in our experiment. The urine output of the individual rats varied somewhat from day to day but it was not uncommon for progesterone treated hypophysectomized animals to excrete during 24



GRAPH 1.
Average daily urine output per rat under the influence of progesterone in hypophysectomized and normal animals.

hours an amount of urine corresponding to half their body weight.

Since the water intake was approximately the same as the output we did not deem it necessary to include the values for water intake in our table.

It seemed of some interest to establish whether increased urine output is merely the result of the increased intake. For this purpose 15 cc of tap water was administered by stomach tube to each rat and then water and food were withdrawn from all animals during 24 hours. Here again the progesterone-treated animals excreted the water more rapidly than the oil-treated controls. This was true both in the case of the intact and the hypophysectomized animals. It is of interest however, that 4 out of 6 oil-treated hypophysectomized controls succumbed from water intoxication after the administration of the relatively large amount of 15 cc while no casualties occurred in any other group. This is in accord with many previous observations made in this laboratory which indicate that progesterone increases the general resistance of hypophysectomized rats.

We wish to call attention to the fact that experiments performed by the senior author in coöperation with Miss C. Dosne indicate that desoxycorticosterone acetate exerts a diuretic effect similar to that of progesterone. These latter observations will be published in detail in the near future.

Summary. Progesterone increases the urine output both in normal and in hypophysectomized rats. Although the untreated hypophysectomized animal excretes less than the normal amount of urine, progesterone stimulates water excretion much more markedly in the absence than in the presence of hypophysis. In hypophysectomized rats, it may lead to the excretion of an amount of urine corresponding to 50% of the experimental animal's total body weight; a diuresis reminiscent of diabetes insipidus. The resistance of hypophysectomized animals to water intoxication is increased by progesterone.

Effect of Volume Used for Injection in Micro-Assay of Prolactin.

ROBERT W. BATES AND OSCAR RIDDLE

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Cold Spring Harbor, N. Y.*

The local crop-sac method, or micro-method, of testing for prolactin (intracutaneous injection over the pigeon's crop) introduced by Lyons and Page¹ involves a subjective element and is open to still other sources of error; it has, however, the advantage of extreme sensitivity. Bates and Riddle² reported that subcutaneous injections over the crop-sac area, though they place the prolactin nearer to the responding tissue, are markedly less effective than similar intracutaneous injections. Since the micro-method is currently used in some laboratories for quantitative assay of prolactin, particularly in blood and urine where the concurrent use of 0.1 and 1.0 ml fluid was once advised,¹ it is desirable to learn the sources of error in the use of this method. The present study is concerned with the effect of injecting equal quantities of hormone in unequal volumes of fluid.

In the local crop-sac method the intracutaneously injected prolactin stimulates that area of the crop tissues which lies immediately beneath the site of injection. This fact suggests a direct diffusion from the injection site. If uncomplicated diffusion is involved one would expect the response to be directly proportional to the concentration of prolactin in the solution injected. The crop epithelium is the tissue upon which prolactin acts (by causing cell proliferation) and in passing from the site of injection to this epithelium the hormone must traverse the following very thin structures: lower dermis; a layer of loose connective tissue and fat lying between the crop and the skin; the serosa; and two thin layers of muscle in the crop wall. The crop epithelium is in actual contact with ingested food and the proliferation induced by prolactin occurs in its basal cells.

When 0.05 ml of liquid is injected intracutaneously over the crop it forms a small disk-shaped blister or bleb of fluid, roughly 5 mm in diameter and 2 mm in thickness. Similarly 0.5 ml of liquid forms a blister 16 mm in diameter and 2 mm in thickness. The relative areas are as 1 : 10, but they have essentially the same thickness. Hence

¹ Lyons, W. R., and Page, E., *PROC. SOC. EXP. BIOL. AND MED.*, 1935, **32**, 1049.

² Bates, R. W., and Riddle, O., *PROC. SOC. EXP. BIOL. AND MED.*, 1936, **34**, 847.

the concentration per unit area may be considered independent of the volume of fluid injected. It is our experience that when stretched for examination the area of crop epithelium stimulated is 5 to 10 mm larger in diameter than the area of the skin displaced by the injected fluid, and that this area is located directly beneath the site of injection. If the mode of transfer of prolactin is by diffusion alone a prolactin solution which produces minimum stimulation when a volume of 0.5 ml is injected should also show minimum stimulation (over a smaller area) when a volume of 0.05 ml of the same solution is injected. The following experiments were designed to test this theory along with a determination of effects of unequal volumes on micro-assay.

Experimental. In tests made with 2 daily injections into the same site 7 groups of 4 birds each were used. Each bird was injected intracutaneously over both crop-sacs with the same amount of prolactin but on one side the volume was 10 times that on the other (0.5 and 0.05 ml); thus the concentration was 10 times greater in the smaller volume of solution. The right crop-sac of all birds of 4 of the 7 groups received the larger volume (Series A, Table 1). The left crop-sac received the larger volume in all birds of 3 other groups (Series B, Table I). The 2 sides of the crop were thus shown to be equally responsive. Each bird was

TABLE I.
Effect of Injection Volume on Response of Crop-sacs to Local Intradermal Injections of Prolactin. Both Sides of Crop (R and L) Injected Twice at 24-hour Interval with Autopsy 48 Hours After First Injection.

Total dose prolactin No. 437	No. of birds	Daily volume, (ml)	Concen- tration, γ/ml	Series	Side of crop used (R or L)	Results	
						No. positive	Sum of plus values
2.0γ	4	.5	2	A	R	4	+12
2.0γ	4	.05	20	A	L	4	+ 6
1.0γ	4	.5	1	A	R	4	+10
1.0γ	4	.5	1	B	L	4	+ 6
1.0γ	4	.05	10	A	L	2	+ 2
1.0γ	4	.05	10	B	R	2½	+ 2½
.5γ	4	.5	0.5	A	R	2½	+ 4½
.5γ	4	.5	0.5	B	L	3½	+ 3½
.5γ	4	.05	5.0	A	L	1½	+ 1½
.5γ	4	.05	5.0	B	R	0	+ 0
.25γ	4	.5	0.25	A	R	3½	+ 6
.25γ	4	.5	0.25	B	L	1½	+ 1½
.25γ	4	.05	2.5	A	L	1½	+ 1½
.25γ	4	.05	2.5	B	R	0	+ 0

injected twice in the same spot and killed 48 hours after first injection. The presence of stimulation was estimated from examination of the stretched excised crop by transmitted and reflected light. Several of the birds were also given colchicine 6 to 8 hours before killing and the presence or absence of accelerated cell division was determined histologically. In all tests the gross degree of stimulation was estimated as plus 4, 3, 2 and 1; $\frac{1}{2}$ was used for cases in which stimulation was doubtful. Degree of stimulation is significant (though arbitrarily determined) and the sum of "plus" values for the 4 birds of each group is tabulated. The value assigned to each individual case was probably affected by the larger area of stimulation which results from the larger volume; we tried, however, to consider thickness only.

Crop-sacs stimulated by 0.5 ml containing 0.125 γ in each of 2 injections (total, 0.25 γ) responded as much on the average as crop-sacs stimulated by 0.05 ml containing 0.5 γ (total of 1.0 γ). The ratio of concentrations in these 2 cases is 40 : 1. Thus one obtains the wholly unexpected result that a dilution of 10 times increased the effectiveness of the injected prolactin by a factor of 4.

In a third series of tests (Table II) 4 groups of 3 pigeons each were treated as in the preceding tests except that the total dose was given in a single injection (and killed after 48 hours). Identical results on the relation of volume to response were obtained although 4 to 8 times as much prolactin was required to produce any particular grade of response with a single injection. One γ in 0.5 ml produced as much stimulation as 4 γ in 0.05 ml; 2 γ in 0.5 ml produced as much stimulation as 8 γ in 0.05 ml. Comparison of "once"

TABLE II.

Effect of Injection Volume on Response of Crop-sacs to a Single Local Injection of Prolactin with Autopsy 48 Hours Later.

Total dose prolactin No. 437	No. of birds	Volume, (ml)	Concen- tration, γ /ml	Side of crop used (R or L)	Results	
					No. positive	Sum of plus values
8.0 γ	3	.5	16	R	3	+ 8
8.0 γ	3	.05	160	L	2 $\frac{1}{2}$	+ 4 $\frac{1}{2}$
4.0 γ	3	.5	8	R	2 $\frac{1}{2}$	+ 4 $\frac{1}{2}$
4.0 γ	3	.05	80	L	1	+ 1
2.0 γ	3	.5	4	R	2 $\frac{1}{2}$	+ 4 $\frac{1}{2}$
2.0 γ	3	.05	40	L	$\frac{1}{2}$	+ $\frac{1}{2}$
1.0 γ	3	.5	2	R	2	+ 3
1.0 γ	3	.05	20	L	0	+ 0

injected groups with "twice" injected groups indicates a quadrupling of effectiveness in the latter case.

Discussion. Our observations do not support the assumption that local stimulation of the crop-sac is due to uncomplicated diffusion of prolactin. The volume of fluid injected seems to have an influence or control on the sensitivity of the reaction and this influence is far greater than that which is expected. This may indicate that, in response to the irritation produced by the injection, some substance released in the skin serves to augment the effect of the prolactin. This assumption is supported by the low effectiveness of prolactin injected subcutaneously over crop-sacs, and by other current observations made in this laboratory which show that substances which irritate or insult the skin at the site of injection will cause some cellular proliferation (confirmed histologically) of the crop epithelium. To date none of those irritating substances induce such proliferation when injected systemically.

Calculations of the relative sensitivity of the local and the systemic methods are of interest. Systemic assays of prolactin No. 437, the preparation used in all our tests, showed it to contain 5 Riddle-Bates (or 5 International) units per mg. This is equivalent to about 1.5 to 2.0 systemic minimum stimulating doses (M.S.D.; 50% positive) per mg in a 450 g pigeon (*i.e.*, 500 to 700 γ = 1 M.S.D.). Using 2 injections, each of 0.5 ml volume, over the crop-sac the M.S.D. of No. 437 was found to be not more than 0.25 γ ; when a single injection was employed 4 times as much prolactin (1 γ) was required. These values thus indicate an increase in sensitivity of at least 500 and 2,000 times for single and double injections, respectively. The magnitude of these increases agrees well with Lyons³ value of 1,000 for a single injection; but it differs greatly from results of Bergman, Meites and Turner⁴ who report an increase of only 178 although they used the still more sensitive 4-day test (of Lyons). From such 4-day tests Lyons⁵ reported an increased sensitivity of 10,000 times.

The importance of volume of fluid injected is not appreciable in systemic injections by the intramuscular route.

Summary. In 2 types (single and double injection) of 48-hour micro-methods for assaying prolactin, in which the injection volume was 0.05 ml, 4 times as much prolactin was required for minimum stimulation as when the volume was 10 times larger, 0.5 ml. A

³ Lyons, W. R., *Proc. Soc. Exp. Biol. and Med.*, 1937, **35**, 645.

⁴ Bergman, A. J., Meites, J., and Turner, C. W., *Endocrinol.*, 1940, **26**, 716.

⁵ Lyons, W. R., *Cold Spring Harbor Symposia on Quant. Biol.*, 1937, **5**, 198.

minimum stimulation dose of prolactin in 0.05 ml thus has its apparent effectiveness increased 4 times by a dilution of 10 times. No simple explanation of this result is apparent. Micro-assays of prolactin by intracutaneous injections over crop-sacs must utilize a constant volume of fluid to be of much quantitative value.

11510

Use of Illuminating Gas to Check Metabolism Apparatus.

IVAN BUNNELL AND FRED R. GRIFFITH, JR.

From the Department of Physiology, University of Buffalo, Buffalo, N. Y.

Combustion of alcohol, ether or acetone is standard procedure to check the operation of metabolism apparatus (Carpenter, *et al.*¹). We have found it impossible, however, to devise a burner by which the combustion of any of these would proceed evenly for metabolically significant periods at rates comparable to the respiratory metabolism of the rat. On the other hand, small validity would seem to attach to a test several times more severe than planned capacity; failure would be no indication of inability of the apparatus to do what it was designed to do; nor would success be any guarantee that it could perform the more delicate task for which it was made. A micro-balance is not checked with kilogram weights.

As a result, recourse has been had to combustion of gas which can be successfully controlled at almost any desired rate. It was originally intended to use a pure, commercial preparation of one of the lower hydrocarbons in order to eliminate the necessity of control determinations. Preliminary work with ordinary illuminating gas from the city mains was so satisfactory, however, that it has been adhered to; especially since equipment was at hand for the necessary control determinations which involve only slightly additional work.

Since the only difficulty in the application of this principle is accurate measurement of the small volume of gas burned, description of an apparatus which has been found accurate and simple to operate and is easily assembled from odds and ends about any laboratory may be of interest.

This apparatus is shown diagrammatically in Fig. 1.

¹ Carpenter, T. M., Fox, E. L., and Sereque, A. F., *J. Biol. Chem.*, 1929, **82**, 335.

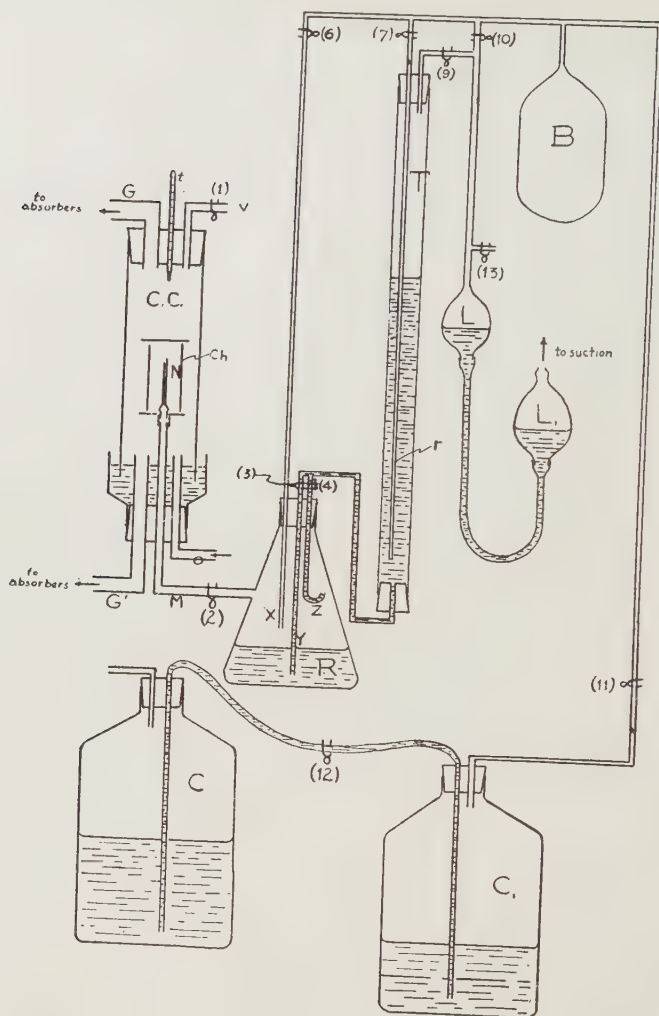


Fig. 1

The gas sample. Since the composition of the city gas supply is not absolutely constant, gas is not burned directly from the supply line. Instead, a sufficient sample for several runs and their attendant control determinations is secured in the 18 liter carboy (C_1) by water displacement into the similar carboy (C). Conversely, as needed, the gas is displaced from (C_1) into the rubber football bladder (B) by siphoning of water from (C) to (C_1).

The combustion chamber, (C-C) 5.5 cm in diameter and 25 cm long, takes the place of the animal chamber of the metabolism

apparatus (Schwabe and Griffith²). The base of the chamber (S) is the upper part of a wide-mouth bottle and contains mercury for an air-tight seal. Tube (O) connects with the oxygen measuring device of the metabolism apparatus and admits pure oxygen as needed. Tubes (G) and (G₁) connect with the carbon-dioxide absorbers of the metabolism apparatus, by which, also, circulation of air within the chamber is maintained. (t) is a thermometer and (V) a tube which is open during assembly of the chamber to equalize

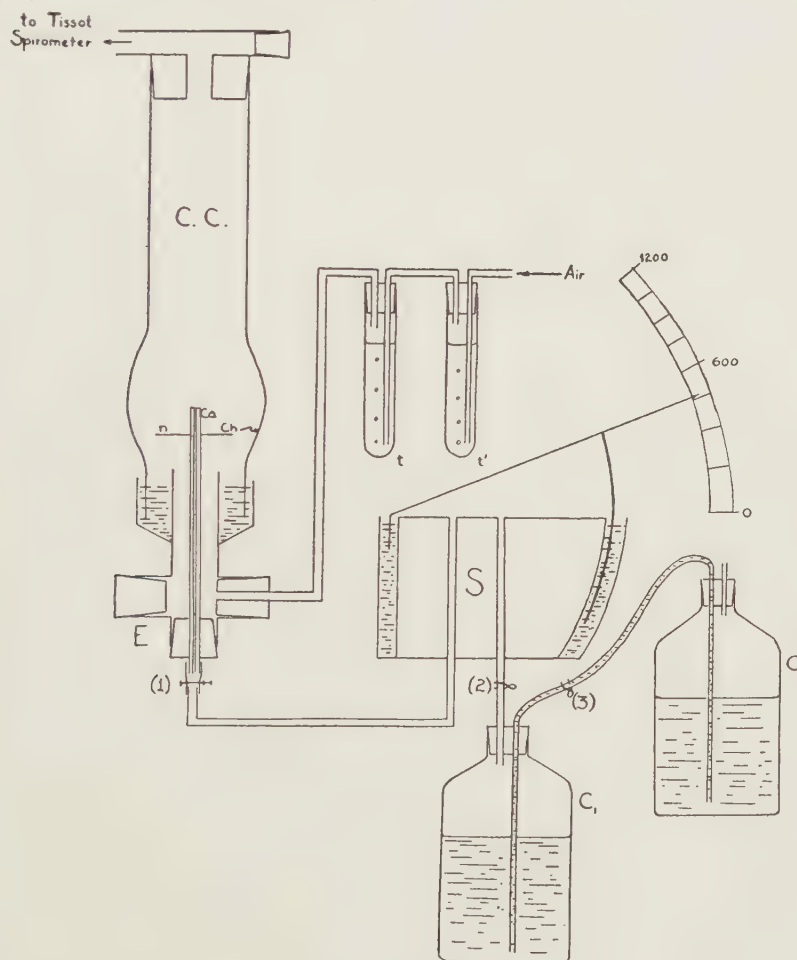


Fig2.

² Schwabe, E. L., and Griffith, F. R., Jr., *J. Nutrition*, 1938, **15**, 187.

pressure. The illuminating gas is admitted through (M) to whose end, within the chamber, a 26-gauge hypodermic needle (N) which serves as burner, is attached by rubber tubing. The burner (and flame) is protected from air-currents by the chimney (Ch).

Gas measurement is made by the assembly consisting of the one-liter suction flask (R) and the tube (T) which is 2.5 cm in diameter and 100 cm long. It is merely an adaptation of the "microspirometer" of Hanan³ similar to that used by Schwabe and Griffith to measure the rate of oxygen utilization in the metabolism apparatus. At the beginning of a run (T) is full of water and (R) has been filled with gas to be burned through tube (X) from the bag (B). During a run, due to the fact that the end of (r) in (T) is 15 cm above the opening of (Z) in (R), water flows from (T) to (R) through (Z), and gas enters (T) from (B) through (r) (Mariotte principle), thus establishing a slight, constant, positive pressure on the gas in (R). This gas is forced from (R) through (M) to (N) at the tip of which it is burned. By proper adjustment of the screw-clamp (4) water can be made to flow from (T) to (R) and gas from (R) to (N), at any desired rate. (T) is carefully calibrated so that the amount of water siphoning into (R) and therefore the volume of gas which has been burned, can be accurately known.

Procedure. At the beginning all clamps are closed except (11) and (12). Since there is positive pressure on the gas sample in (C₁) this will fill the football bladder (B). Also with clamps (6) and (2) open and the combustion burner (N) open to the room, (R) is thoroughly flushed out to make sure it contains no trace of foreign gas. Clamps (2) and (6) are then closed, as well as (11) and (12).

To set (R-T) for a run, *i.e.*, to get the water from (R) to (T) a negative pressure is established by the leveling bulbs (L) and (L₁). Open (13) and raise (L₁) until (L) is full; close (13). Lower (L₁) below (L); this establishes a negative pressure in (L) which upon opening clamps (9), (3) and (6) will draw water from (R) to (T). Actually, since (T) is 100 cm long, sufficient negative pressure to effect this transfer is obtained with the help of a water-faucet suction pump attached to the upper, open end of (L₁). When this transfer is complete (9) is closed, the suction is stopped and (3) and (6) are closed.

By operating the unit (T-R) as a closed system as is done here and as explained by Schwabe and Griffith who used a similar modification of Hanan's device for measurement of oxygen admitted

³ Hanan, E. B., *Science*, 1929, **70**, 582.

to the metabolism chamber, the water that is employed is kept in constant contact and equilibrium with the gas that is used, thereby preventing error due to solution of the gas in the water.

At this time all clamps are closed.

To prepare for a run, clamps (7), (4) and (2) are opened in this order. Due to the slight positive pressure between (T) and (R), water will immediately begin to siphon from (T) to (R); gas will enter (T) from (B) at atmospheric pressure and will escape from the burner (N) where it is lit. The chimney (Ch) is put in place, the line to the oxygen supply opened, and with clamp (1) open the combustion chamber is put in place. Before closing (V) slight suction is applied to it to draw enough pure oxygen into the chamber to make sure the flame will burn; clamp (1) is then closed.

Control determination is effected by the set-up shown in Fig. 2. A sample of the same gas is transferred from the carboy (C_1) to the small one-liter, Krogh spirometer (S) and burned at the tip of the 1 mm capillary glass tube (Ca) in the combustion chamber (CC). The flame is protected by the baffle (n) from the current of air (from the compressed air supply) which enters the combustion chamber after being washed through NaOH ($t-t_1$) and

TABLE I.
Results.

		Rate gas burned, cc/min	R.Q.	Ratio CO ₂ /gas	Ratio O ₂ /gas	Rate of O ₂ utilization, cc/min
1/9/40	E*	5.0	.51	.82	1.60	8.00
	C†		.49	.79	1.60	
1/9/40	E	10.0	.52	.82	1.58	16.00
	C		.49	.79	1.60	
1/22/40	E	5.5	.51	.84	1.68	9.25
	C		.52	.87	1.66	
2/5/40	E	5.1	.49	.79	1.57	8.01
	C		.50	.83	1.68	
2/13/40	E	5.2	.49	.85	1.72	9.15
	C		.50	.85	1.71	
4/22/40	E	6.5	.52	.84	1.62	10.53
	C		.50	.86	1.73	
Average	E		.51	.83	1.63	
	C		.50	.83	1.66	

*Refers to results obtained when a small flame was burned in the Schwabe-Griffith Metabolism Apparatus.

†Refers to results obtained when the products of combustion of a larger flame were determined with the Tissot spirometer and gas analysis.

on leaving (at the top of CC) is collected and measured in a 100-liter Tissot spirometer. This air is then analysed in Haldane apparatus.

Comparison of the results obtained in a few of the more recent "experimental" and "control" determinations is shown in Table 1.

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Studies on Intercostal Nerve Physiology.

ALFRED J. KAHN. (Introduced by Arno B. Luckhardt.)

From the Hull Physiology Laboratory, University of Chicago.

Pathological cases of processes involving the parietal pleura and the diaphragmatic pleura innervated by the lower intercostal nerves, and the attendant referred pain and muscular rigidity in the abdominal region (lower quadrant), have long suggested neural associations between the regions involved. Capps¹ competent studies upon such clinical cases have yielded valuable information relating the portion of pleural membrane or diaphragm stimulated to site of referred pain.

It is a well established fact known to clinicians that:² "The abdominal symptoms of thoracic disease are often so misleading that needless or harmful surgery may be carried out. A pleurisy may give referred pain over the distribution of the 6 lower intercostal nerves, with fever may simulate appendicitis."

In attempting to establish a physiological basis for such phenomena, we have investigated the intercostal nerves of dogs. The effects of our various manipulations upon blood pressure and respiration were kymographically recorded, the blood pressure obtained from the carotid artery, and respiratory movements recorded by the usual pneumograph-tambour method. In cases where a pneumo-thorax was unavoidable, artificial respiration was administered. Isolation of various intercostal nerves and their branches was facilitated by appropriate rib resection. A tetanizing current was used for nerve stimulation.

Stimulation of the central end of any intercostal nerve results in a transient lowering of blood pressure (approximately 25 mm Hg.)

¹ Capps, J. A., *An Experimental Study of Pain*, Macmillan, 1932.

² Nelson, *Living Medicine*, Thomas Nelson and Sons, 1920-1937, 5, 222.



FIG. 1.
Effects of stim. of central end of
intercostal nerve VI.

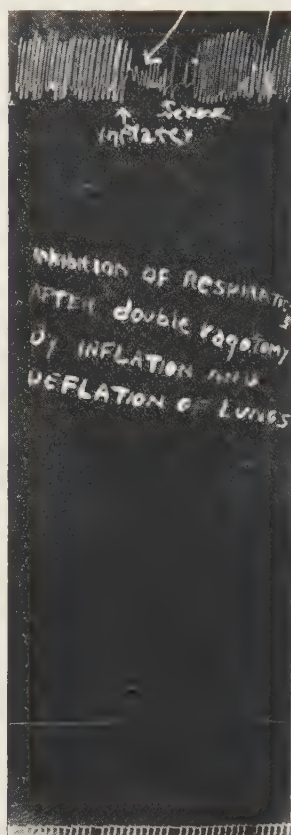


FIG. 2.
After double vagotomy. Inhibition
of respiration by inflation and deflation
of lungs. Arrow points to artifact of
art. resp.

and an inhibition of respiration for a short interval of time. The interval of respiratory inhibition varied in length from 3 to 5 seconds. Normal respiration is resumed after this interval in spite of continuous stimulation. In some cases a decided decrease in amplitude of respiratory excursions was the only respiratory change observed. It was found that the lower intercostal nerves gave rise to more marked respiratory effects than the upper ones.

In attempting to relate the aforementioned effects to specific branches of the intercostal nerves, it was found that the effects on blood pressure and respiration may be elicited by stimulating the central ends of intercostal branches innervating the diaphragm, parietal pleura, and rectus abdominis muscle.

Stimulation of the central end of a branch of the intercostal innervating the diaphragm elicits a *spasm of the rectus abdominis muscle*. Since this reflex is also elicited by stimulation of the central end of the main trunk of a lower intercostal nerve (6-12), and the reflex is not abolished until all of the lower intercostals are severed, regardless of the order in which they are severed, the efferent fibers of the reflex arc must traverse several of the lower intercostal nerves (trunks X-XII especially). This striking reflex is undoubtedly the cause of the increased tonus of the rectus abdominis muscle observed in many cases of lobar pneumonia and pleurisy.

The effect of the parietal pleural nerve fibers upon respiration suggests a normal regulatory mechanism of respiration caused by the mechanical rubbing of the 2 pleural membranes during respiratory movements. Such a regulatory mechanism was also suggested by the work of Scott, Gault, and Kennedy³ who recorded action potentials from the peripheral end of an intercostal nerve during chest expansion. Pike and Coombs⁴ likewise obtained evidence pointing to a regulatory mechanism of respiration by thoracic and cervical dorsal nerve root section. We sectioned the vagi and phrenic nerves and strongly inflated the lungs with air, whereupon respiration ceased for about 20 to 25 seconds. After respiratory movements had started, the lungs were allowed to deflate, and another inhibition of respiration ensued, due to the mechanical stimulation of the parietal pleura by the deflating lungs. The extent of this inhibition with double vagotomy and phrenicotomy was approximately 10-15 seconds. Inflating a balloon in the chest cavity and thus expanding the chest wall internally results in the same respiratory effects. When the phrenic nerves are allowed to remain intact, the intervals of inhibition are greatly reduced (2-5 seconds), and a high cord section (at first thoracic segment) completely abolishes the inhibition. The effect of the phrenic nerves is due to the afferent fibers in them increasing the respiratory rate, as shown by Robb and Deason.⁵

We believe that the intercostal nerves reinforce the Hering-Breuer reflex in normal respiration. The intercostal nerves may also be a factor in regulating the rate of the respiratory excursions with changes in amplitude, that is, the greater the stimulation of the intercostal fibers, the greater the interval to the next respiratory movement.

³ Scott, Gault and Kennedy, *Am. J. Physiol.*, 1922, **59**.

⁴ Pike, F. H., and Coombs, Helen C., *Am. J. Physiol.*, 1922, **59**.

⁵ Robb and Deason, *Am. J. Physiol.*, 1911, **28**.

Summary and Conclusions. 1. Stimulation of the central end of any intercostal nerve causes a reflex inhibition of respiration and effects a concomitant drop in blood pressure. 2. The lower intercostal nerves (7-12) elicit a greater response than do the upper ones. 3. Stimulation of the intercostal branches to the parietal pleura, diaphragmatic pleura, and rectus abdominis muscle give the respiratory inhibition and lowered blood pressure (approximately 25 mm of Hg.) 4. Stimulation of sensory or intercostal fibers in the diaphragm causes reflex contraction of the abdominal musculature through reflex connection with other lower intercostal nerves. 5. Among other things, these results furnish the physiological mechanisms involved in referred pain and muscular rigidity in the lower abdominal quadrant as a result of involvement of the base of the lungs, in lobar pneumonia for example. 6. Both expiration and inspiration cause the intercostal nerves to be stimulated and thereby effect reflexly respiratory inhibition. a. Inspiration more strongly inhibits respiration than does expiration. b. At the end of inspiration, the intercostal nerves aid the Hering-Breuer reflex. c. After expiration the intercostal nerves constitute a factor that determines lapse of time before the next inspiration. 7. Abnormal respiration and tightening of the abdominal musculature may be indicative of an irritation in the peripheral region of the diaphragmatic pleura.

I hereby wish to acknowledge that Dr. Arno B. Luckhardt suggested the problem and rendered valuable aid in its prosecution.

11512

H Ion Concentration of Various Fluids of the Genital Tract of the Cow.

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In a study of certain reproductive phenomena in dairy cattle a few questions were raised which made it necessary to determine the pH of various fluids of the genital tract of the cow. This problem is of considerable scientific and practical interest since it is reported by Warren¹ and others that sex can be controlled by the simple ex-

¹ Warren, Carl, *Animal Sex Control*, Orange Judd Co., 1940.

TABLE I.
The pH of Various Fluids of the Cow.

Fluid	No. of cases	pH	
		Range	Avg
Vaginal douche (Anestrus)	3	6.0-6.7	6.4
Cervical fluid	17	7.6-8.9	8.33
Uterine wash*	13	6.6-7.15	6.8
Amniotic fluid from calf fetus	6	7.0-7.4	7.12
Follicular fluid	3	7.52-7.7	7.6

*5 cc double distilled water was washed through the uterus and the pH of the wash determined.

pedient of acid or alkaline vaginal douches at the time of breeding. The fluids in our studies were obtained as follows:

Specimens of vaginal and cervical fluid were collected from cows in the University herd by means of a speculum and pipette. The other fluids were obtained from cows immediately after slaughter. The pH determinations were made with the Coleman glass electrode apparatus.

In the cow there is very little vaginal secretion during anestrus. The data obtained upon the pH of the various portions of the genital tract of the cow are summarized in Table I.

During estrus a cord of heavy gelatinous mucus is secreted from the cervix into the vagina. This secretion is distinctly alkaline with an average pH of 8.3 and as it flows into the vagina it changes the reaction in that organ to a slightly alkaline one. For this reason the vagina of the cow in estrus is alkaline. These observations support the findings and hypotheses of McNutt² *et al.* This reaction is quite different from the reaction found in the rat, according to Beilly,³ where the vaginal fluid is most acidic during estrus.

From the data given in Table I the sequence of events in the impregnation of the cow would indicate that the sperm are ejaculated into an environment with a slightly alkaline reaction. From there the sperm must pass through the cervical gateway in a medium whose pH normally lies between 8.0 and 9.0. After passing through the cervix the sperm arrives in the uterus which maintains an environment at an average pH of 6.8. This is the pH range which we have found to be optimum for bull sperm storage.⁴ Thus all sperm on an impregnation journey in the normal cow pass through an alkaline bath before arriving in the uterus where a more optimum pH prevails. The pH of solutions of pure sodium bicarbonate is

² McNutt, S. H., Schwarte, L. H., and Eveleth, D. F., *Cornell Vet.*, 1939, **29**, 415.

³ Beilly, J. S., *Endocrinology*, 1939, **25**, 275.

⁴ Phillips, P. H., and Lardy, H. A., *J. Dairy Sci.*, 1940, **23**, in press.

about 8.8 which lies within the range of alkalinity of the cervix during heat. Furthermore, 10 cc of cervical fluid with a pH of 8.3 requires 3 to 4 cc of N/10 HCl to bring it to pH 6.5. Thus the advocates of pH controlled vaginal douches for the control of sex must go beyond the cervix to make their procedure effective in the female bovine.

Summary. A study of the pH of the genital secretions of the cow has shown the vagina to be slightly acid in anestrus and slightly alkaline in estrus due to the distinctly alkaline character of the cervical discharge at that time. The fluids of the cervix during estrus average pH 8.3 with a range from pH 7.6-8.9. The fluids present in the uterus during estrus have an average pH of 6.8 which is a favorable one for sperm survival.

11513

Lessened Effectiveness of Bacteriostatic Agents vs. Tuberculous Infection in Rabbits with Impaired Functional Efficiency.

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This is a report of a study of the effect of administered sulfanilamide (SA) and *p*-caproylaminobenzenesulfonylhydroxamide (CH)* on the rate of increase in size of the local lesion produced by subcutaneous injection of a suspension of avian tubercle bacilli, in the rabbit, and on the bearing—on that effect—of the functional status of the rabbit as indicated by its reaction to chilling.^{1, 2}

Table I indicates the comparative intensity of the growth-restraining effect exerted by SA and CH in cultures of the avian tubercle bacillus in broth. The strain used was *M. avium* No. 30, from the Phipps Institute. It formed smooth, moist, cream-colored colonies on Lowenstein's medium. In broth, it grew in sedimenting, flocculent masses which formed an even suspension on agitation. The culture medium was glycerine veal heart infusion broth of pH 6.8, distributed in 25 cc amounts in 50 cc Erlenmeyer flasks. The

* Supplied by Dr. Maurice Moore, Sharp and Dohme, Philadelphia.

¹ Locke, A., *J. Infect. Dis.*, 1937, **60**, 106; *J. Immunol.*, 1939, **36**, 159; Locke, A., and Main, E. R., *Ibid.*, 173.

² Locke, A., Locke, R. B., Bragdon, R. J., and Mellon, R. R., *Science*, 1937, **86**, 228; Locke, A., Main, E. R., and Mellon, R. R., *J. Immunol.*, 1939, **36**, 183.

TABLE I.
Comparative Effects of Sulfanilamide (SA) and *p*-caproylamino benzenesulfonhydroxamide (CH) on the Growth of the Avian Tubercle Bacillus in Broth Culture.

Series	Total No. cultures in group	Drug added	Conc. millimolar	Avg vol of sediment from 10 cc of culture at 20 days, cc \times 1000	Estimated avg % inhibition
2, 3, 5b	15	none		21	
(small inocula)	5	CH	.26	<1	97
	14	CH	.52	<1	
	6	SA	.26	2	93
	9	SA	.52	<1	
1, 4, 5a	18	none		24	
(heavier inocula)	7	CH	.26	8	60
	8	CH	.52	11	
	7	SA	.26	20	20
	11	SA	.52	19	

drugs were added in 0.1 and 0.2 cc volumes of 60% ethyl alcohol solution. Equivalent additions of 60% alcohol free of dissolved drug produced no recognized growth-checking effect. Ten cc aliquots of the cultures were centrifuged after incubation at 38°C for 20 days. The collected sediments were found to be pure.

The series in the lower group in Table I were inoculated from 3 to 5 times more heavily than those in the upper group. The average percent inhibition exerted by the CH in the upper group was 97; that exerted by the SA, 93. The equivalent averages for the group receiving the larger inocula were 60 and 20. The CH would appear to have been, in this *in vitro* comparison, more effective than the SA but less effective against a relatively large inoculum than against a small one. CH is, mole for mole, also more effective than SA in checking growth of the type I pneumococcus in broth culture but is less effective than SA in cultures containing blood.³ It has approximately the same effectiveness as SA in pneumococcus-infected mice.⁴

The usefulness of the reaction to chilling as an index of functional status, in the rabbit, was established in an investigation of factors determining non-specific capacity for resistance to pneumococcal invasion.¹ Rabbits with "warming times" of 30 to 33 were found to have a maximum non-specific capacity for surviving such

³ Main, E. R., Shinn, L. E., and Mellon, R. R., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 593.

⁴ Cooper, F. B., Gross, P., and Lewis, M., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 491.

TABLE II.

Comparative Size of the Local Lesion Developed Following Subcutaneous Injection of a Suspension of the Avian Tubercle Bacillus into Treated and Untreated Rabbits Making Optimal and Sub-optimal Reaction to Chilling.

Warming time ¹	Rabbit No.	Drug given	Per diem wt loss in g during treatment	Approx. area of lesion in cm ² on the indicated day of infection				
				9	20	40	60	
30-40		Treatment begun immediately after infection.						
	3	none		5	5	7		
	1	none		8G	8G	6GD		
	671	CH	5	1	1*	(9)	(6D)	
	679	CH	0	1	2*	(6)		
	2	SA	23	4*	(13)	(13)		
	675	SA	63	4M				
>40	681	none		12	12	10D		
	677	none		9G	18G	20GD		
	667	CH	14	11*	(14)	(20GD)		
	664	SA	13	7*	(9)	(12)		
30-40		Treatment begun 20 to 40 days after infection.						
	656	none			8	2D	3D	
	639	CH	4		8†	10		
	649	CH	0		4†	4	0‡	
	655	CH	0			6†	6D	

*Day of discontinuance of treatment. The subsequent, bracketed values indicate extent of change in lesion size following cessation of treatment.

†Day treatment was begun.

‡Excision of the area revealed persisting tubercle bacilli. The notation M means dying. The terminal blood SA level, in this rabbit, 18 hr after the last preceding SA feeding, was 28 mg%. G denotes lymph-node invasion and D, drainage.

invasion and rabbits with warming times appreciably longer than 40, a minimum capacity for survival. Transition from a warming time of 40 or longer, to 33, could be induced through enforced rest, circulatory support and vitamin administration; and converse impairment from 33 to more than 40 through exhaustion, shock and starvation. The ability of SA to check pneumococcus invasion in rabbits with warming times less than 40 was observed to be more than double that observed in rabbits with warming times exceeding 40.²

The warming times of the upper group of rabbits in Table II lay between 30 and 40. Those of the lower group lay beyond 40. New Zealand White males were used, from 3 to 4 kg in weight. They were injected subcutaneously, within a shaved area over the abdomen, with 0.002 to 0.004 cc of sedimented, growing avian tubercle bacilli suspended in 0.5 to 0.75 cc of broth, one or more days after the last preceding estimation of warming time and from 0 to 2 hours before beginning drug feedings. The SA was given in capsule in a dosage of 0.3 g 8 x daily and the CH in a dosage

of 0.45 g 5 x daily or 0.23 g 8 x daily. Preliminary trial had indicated the ineffectiveness of smaller, less frequent dosage. The drugs could not be given, successfully, in admixture with food.

The infection was not rapidly fatal. The indicated death of rabbit 675 was due to sulfanilamide poisoning, not infection. The SA proved to be so toxic in effective dosage as to prohibit sustained use. Loss of appetite was induced, together with rapid decrease in weight. The comparative non-toxicity of the CH was the determining point in its choice for this study. CH produced no impairment in appetite, significant loss of weight or other directly recognizable injury during sustained, massive feedings over periods of 20 to 40 days. Adjuvant feedings were given, during protracted CH administration, of sodium bicarbonate, ascorbic acid and liver extract.

The lesions produced at site of injection in the control rabbits with warming times of 30 to 40 were, roughly, one half the size of those developed in the control rabbits with warming times longer than 40. A comparable relationship was apparent in the treated rabbits following cessation of treatment. During the period of treatment, the lesions in the 30-40 minute rabbits given CH from the outset of infection were approximately one-sixth the size of those observed in the controls but quickly grew out to normal size following discontinuance. The corresponding lesions in the 30-40 minute rabbits given SA were about two-thirds the size of those observed in the controls. No significant effect of CH or SA was indicated in the rabbits with warming times exceeding 40, either on a basis of lesion size at time of drug discontinuance or on a basis of extent of further development following withdrawal. CH administration begun 20-40 days after infection was without significant checking effect. The infection was in no case eradicated.

Summary. Sulfanilamide and *p*-caproylaminobenzenesulfonhydroxamide, producing 20 and 60% inhibition of growth, respectively, in broth cultures of the avian tubercle bacillus, exert a comparable degree of restriction on the rate of increase in size of the local lesion produced by subcutaneous injection of this organism, in rabbits with warming times less than 40, but not in rabbits with warming times appreciably longer than 40.

11514

Sulfapyridine and Serum in Experimental Type III Lobar Pneumonia.

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Previous experiments¹ have demonstrated that sulfapyridine and sulfanilamide were about equally effective in reducing mortality in Type III lobar pneumonia of rats. Up to the time of publication of those results, the samples of specific antiserum that we had tested had shown no decisive effects in protecting against Type III infections in rats. Within the last year accurately standardized, highly concentrated Type III serums* have been available and in our experiments, when used in large doses, they have exhibited a protective value approaching that of type specific serum in Type I pneumococcic pneumonia.²

In these experiments serum and sulfapyridine† were used together and separately in doses which had been found to afford the greatest degree of protection. The optimal quantity of serum was found to be between 1,000 and 2,000 units per rat when the total was distributed over a period of 7 days and that for sulfapyridine between 850 and 1250 mg when given in divided doses over the same period.

One hundred and sixty young adult rats weighing between 140 and 280 g were infected in groups of 40 by the intrabronchial method previously described by Nungester and Jourdonais.³ In each group there were 10 untreated controls, 10 treated with serum by intraperitoneal injection, 10 treated with sulfapyridine, administered by stomach tube, and 10 treated with both serum and sulfapyridine.

The strain of Type III pneumococcus was that used in previous experiments.¹ The dose used in the first 2 groups of 40 rats was 0.1 cc of a 16-hour bouillon culture, diluted to 10^{-5} . This produced in the untreated controls a mortality of 63%. In the third and fourth groups the dose was increased 10 times (10^{-4} dilution) and the initial mortality was 100%.

Treatment was begun in all cases about 4 hours after injection.

* Donated by Lederle Laboratories, Inc., New York, N. Y.

† Donated by Merck and Company, Rahway, N. J.

¹ Kepl, M., and Gunn, F. D., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 457.

² Kepl, M., and Gunn, F. D., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **40**, 529.

³ Nungester, W. J., and Jourdonais, L. F., *J. Bact.*, 1935, **29**, 34.

TABLE I.
Sulfapyridine and Serum in Experimental Type III Lobar Pneumonia.

Total No. of rats	No. survivors	No. fatalities	% mortality	Therapy	Dilution of organisms
19*	7	12	63	None	1:100,000
20	13	7	35	Serum	1:100,000
19*	14	5	26	Sulfapyr.	1:100,000
19*	14	5	26	Serum and Sulfapyr.	1:100,000
20	0	20	100	None	1:10,000
20	4	16	80	Serum	1:10,000
18*	11	7	39	Sulfapyr.	1:10,000
16*	10	6	37	Serum and Sulfapyr.	1:10,000

*In each group 20 animals were originally injected. However, those killed by trauma incurred by the injection of the drug, or those which did not show the ink spot tracer in the lung on post-mortem examination, were deleted from the experiment.

The initial dose of sulfapyridine was 250 mg, emulsified in 2 cc of 1.5% mucin. This was followed by a twice daily dose of 125 mg for 2 days and then 125 mg daily for 4 more days. In the first 2 experiments the initial dose of serum was 250 units in 1 or 2 cc of saline and the maintenance dose was 125 units given twice daily on the second and third days and once a day for the next 4 days. In the third and fourth experiments the method differed only in that the initial dose was 500 units instead of 125 units.

In the table, the first and second groups and the third and fourth groups are combined. Serum therapy alone resulted in a reduction of mortality from 63% to 35% in the 80 rats which were infected with the smaller dose of pneumococci. Under the same conditions, animals receiving sulfapyridine alone showed a mortality of 26%. The same figure is shown for the group receiving both serum and sulfapyridine.

In the 80 rats which were infected with the larger dose (10^{-4} dilution) serum therapy reduced the mortality from 100% to 80%; sulfapyridine therapy reduced it to 39%. A combination of sulfapyridine and serum showed no significant improvement over sulfapyridine therapy alone, giving a mortality of 37%. Under the second group of conditions, where the most important factor which has been changed is the dose of pneumococci (increased from a dilution of 10^{-5} to 10^{-4}) sulfapyridine appears to be about 3 times as effective as serum (survival rate of about 60% for sulfapyridine and about 20% for serum). In unpublished experiments we have found that a further increase of the dose of serum, especially in the first 2 days, does not further reduce the mortality when the infecting dose is large.

Summary. Under the conditions of our experiments the protective value of highly concentrated type specific serum in optimal doses and that of sulfapyridine in optimal doses are approximately equal when the infecting dose of Type III pneumococci is relatively small, resulting in a mortality of 63% in untreated animals. When the infecting dose is sufficiently large to produce an initial mortality of 100%, the mortality after sulfapyridine therapy is significantly less than after serum therapy. Combining serum and sulfapyridine, each in optimal dose, does not reduce mortality below that of sulfapyridine therapy alone in Type III pneumococcic pneumonia, differing in this respect from results previously obtained from similar experiments with Type I pneumococcic pneumonia.

11515

Effect of Sulfanilamide, Sulfapyridine, and Sulfathiazole on Staphylococcus Toxins.*

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Conflicting reports have appeared in the literature on the neutralization of staphylococcus toxins by sulfanilamide and allied compounds. Levaditi and Vaisman¹ were unable to demonstrate any effect of prontosil, neoprontosil, and other azo-sulfonamide derivatives against staphylococcal hemolysin, although they claimed these compounds neutralized the effect of streptococcal leucocidin and hemolysin. Later Levaditi, Vaisman, and Reinie² reported that none of the compounds tested was effective against staphylococcus lethal toxin. Osgood and Powell³ found that sulfanilamide in concentrations of 1:1000 or less did not inactivate significant amounts of staphylococcal hemolysin. Recently Carpenter and his co-

* The author wishes to acknowledge his appreciation of the interest, and the valuable suggestions of Dr. E. K. Marshall, Jr., and Dr. Perrin Long.

¹ Levaditi, C., and Vaisman, A., *Compt. Rend. Soc. de Biol.*, 1935, **120**, 1077.

² Levaditi, C., Vaisman, A., and Reinie, L., *Compt. Rend. Soc. de Biol.*, 1937, **126**, 1937.

³ Osgood, E. E., and Powell, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1938, **39**, 37.

workers⁴⁻⁷ have reported an antitoxic effect of sulfanilamide and its derivatives on toxins formed by the gonococcus, pneumococcus, staphylococcus, *Streptococcus hemolyticus*, *Clostridium botulinum*, *Clostridium tetani*, *Clostridium septicum* (vibrio septique), and *Clostridium perfringens*. Although in his *in vivo* experiments he reports in one paper⁵ neutralization, and in another paper,⁷ failure to neutralize staphylococcus lethal toxin, he reports statistically valid *in vitro* experiments demonstrating consistently the neutralization of staphylococcus lethal toxin.

In the present study, toxins from 4 strains of hemolytic staphylococci were used, as well as one batch of toxin labelled "Lot O" prepared by Carpenter. The effect of sulfanilamide (para amino benzene sulfonamide), sulfapyridine (2-sulfanilyl amino pyridine), and sulfathiazol (2-sulfanilyl aminothiazole) on these toxin preparations was investigated. The toxins were prepared according to the method of Dolman and Wilson.⁸ A 2-day culture of the organism following growth on semi-solid agar was passed through filter paper, and finally through a Seitz filter to remove the bacteria. The M.L.D. of each lot of lethal toxin was determined for adult mice weighing 22 to 26 g. In this study two strains of albino mice and one strain of black mice were used, but only one strain of mice was used in each experiment. Each group, including the control group, contained the same proportion of males and females with comparable weights. The mice were observed for 7 days, although very few deaths occurred after 48 hours. The sulfanilamide or its derivative (in 0.85% sodium chloride solution) was thoroughly mixed with the toxin and 1 cc of the mixture injected intraperitoneally into each mouse from 5 to 45 minutes following mixing. Control mice received 1 cc of the saline solution containing the same amount of toxin.

During initial experiments in which a dose of toxin was administered sufficient to kill 100% of the control mice, it was always found that 100% of the mice injected with toxin-sulfanilamide mixtures also died. Subsequent experiments were therefore per-

⁴ Carpenter, C. M., Barbour, G. M., and Hawley, P. L., *J. Pediatrics*, 1939, **14**, 116.

⁵ Carpenter, C. M., and Barbour, G. M., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 354.

⁶ Carpenter, C. M., and Barbour, G. M., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 255.

⁷ Carpenter, C. M., *Proc. Third International Congress for Microbiol.*, 1939, **7**, 595.

⁸ Dolman, C. E., and Wilson, R. J., *J. Immunology*, 1938, **35**, 13.

TABLE I.
Effect of Sulfanilamide and Allied Compounds on Staphylococcus Lethal Toxin.

	Mice injected		% survival
	Total	No. survived	
Control	150	79	56
1:100 Sulfanilamide	100	36	36
1:200 "	100	39	39
1:1000 "	120	55	45
1:1000 Sulfapyridine	120	58	48
1:1000 Sulfathiazole	120	61	51

formed in which approximately half of the control mice survived. Table I summarizes the results of the latter experiments. It is evident that rather than neutralizing the lethal action of the toxin, if anything the drugs slightly enhanced the toxicity to mice. It is difficult to reconcile these results with those of Carpenter. It was therefore thought desirable to determine whether these compounds might influence the other toxic activities of staphylococcus toxin.

The neutralization of dermo-necrotic toxin was tested by injecting 4 albino guinea pigs and 4 albino rabbits with staphylococcus toxin plus various dilutions of sulfanilamide and its derivatives. Each animal was injected intradermally with .2 cc of each mixture. The area of necrosis was measured on the fifth day. The average of these measurements is listed in Table II.

It is obvious that there was no neutralization of the dermo-necrotic factors present in the toxin.

The effect on the hemolysins was studied by several methods. Using 2 hemolytic units of toxin with the addition of various concentrations of the sulfonamides to a 1% rabbit erythrocyte suspension, it was found that high concentrations of the chemicals reduced the hemolysis partially, whereas concentrations normally attained in the blood during treatment of infections had no effect. This

TABLE II.
Effect of Sulfonamides on Dermo-necrotizing Toxin.

	Avg of areas of necrosis, cm ²
Toxin + saline solution (control)	2.2
" + 1:10,000 sulfanilamide	2.0
" + 1: 1,000 "	2.2
" + 1: 100 "	2.3
" + 1:10,000 sulfapyridine	2.2
" + 1: 1,000 "	2.1
" + 1:10,000 sulfathiazole	2.2
" + 1: 1,000 "	2.0

TABLE III.
Effect of Sulfonamides on Alpha-Hemolysin.Rabbit erythrocytes 1% + 2 hemolytic units staphylococcus hemolysin incubated
1 hour 37°C.

Concentration, %	.1	.05	.025	.0125	.006*	.0002*	Control
Sulfanilamide	2+	3+	3+	4+	4+	4+	4+
Sulfapyridine			2+	3+	4+	4+	4+
Sulfathiazole			2+	3+	4+	4+	4+

*Concentrations of .003, .0015, .0008, and .0004 gave same results as .006 and .0002.

confirms the report of Osgood and Powell,³ and of Gross, Cooper, and Lewis.⁹ Similar experiments, using sheep erythrocytes with incubation at 37°C for one hour followed by 12 hours at 7°C, gave essentially the same result.

Blood agar plates were prepared containing various concentrations of the 3 chemicals. The results in Table IV demonstrate that decreased production of hemolysin by staphylococci is due mainly to a decreased growth rate.

In a series of experiments to determine the effect of these compounds upon staphylococcus enterotoxin, no demonstrable neutralizing activity was found. Kittens injected intraperitoneally with toxin-sulfanilamide mixtures vomited in the same length of time, and with the same degree of severity as kittens injected with toxin alone.

Using dilutions of 24 hour broth cultures of various strains of staphylococci mixed with various concentrations of the 3 compounds, no significant alteration of coagulase other than might be attributed to reduced growth rate of the organism was observed.

Although numerous statements have been made concerning the mechanism by which sulfanilamide acts, there is very little proof in support of any of these, other than those concerning its bacteriostatic action. The experiments described in this report were performed in the hope that the neutralization of the toxic products

TABLE IV.
Effect of Sulfonamides on Hemolysin Production in Blood Agar Plates.

Concentration %	Control	Sulfanilamide			Sulfapyridine		Sulfathiazole	
		.01	0.1	1.0	.01	0.1	.01	0.1
Diameter hemolysis (mm)	4.5	3.3	2.1*	0.3*	2.8*	2.2*	2.8*	1.2*
Diameter colony (mm)	1.6	1.5	1.0	0.2	0.8	0.7	1.0	0.7

*Indicates partial hemolysis.

⁹ Gross, P., Cooper, F. B., and Lewis, M., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 275.

might be one of the main activities of the sulfonamide compounds, and that more potent antitoxic compounds might prove even more useful in the treatment of bacterial infections. These experiments, however, lead one to believe that the only manner in which the toxicity of staphylococci is affected is by an inhibition of growth of the organism with a consequent decreased production of toxin.

Summary. Toxic manifestations of staphylococci are not inactivated *in vitro* by sulfanilamide, sulfapyridine, or sulfathiazole. The lethal toxin, dermo-necrotic toxin, coagulase, and enterotoxin are not neutralized by solutions of the sulfonamides tested at 37°C. α - and β -hemolysins are slightly diminished in activity at concentrations approaching the saturation point of the sulfonamides, but are unaffected at concentrations of less than .01%. These compounds appeared to decrease hemolysin production by decreasing the growth rate of the organism.

11516 P

Response of Plasma Volume to Diuretics.

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Previous work¹ has led us to conclude that mercurial diuretics act by diminishing tubular reabsorption, while administration of aminophyllin produces an increase in the volume of the glomerular filtrate. Earlier work² suggested a high circulating blood volume in congestive heart failure, but there was no complete agreement as to the change following diuresis. Recent determinations³ by a more satisfactory method⁴ demonstrate a marked elevation of blood and plasma volume in patients with failure, and a decrease towards normal with the development of circulatory compensation. A similar decrease has been noted after the use of mercurial diuretics.⁵

We have followed over 12 to 24 hours the changes in plasma volume, determined by the method of Gibson and Evelyn,⁴ after the

¹ Herrmann, G., and Decherd, G., *J. Lab. and Clin. Med.*, 1937, **22**, 767.

² Goldhammer, S., Leiner, G., and Scherf, D., *Klin. Woch.*, 1935, **14**, 1109.

³ Gibson, J. G., and Evans, W. A., *J. Clin. Invest.*, 1937, **16**, 851.

⁴ Gibson, J. G., and Evelyn, K., *J. Clin. Invest.*, 1938, **17**, 153.

⁵ Harris, Alfred W., personal communication.

TABLE I.
 Plasma Volume After Injection of Diuretics.

Hr after drug inj.	Plasma vol, cc	Urine flow, cc/min	Plasma vol, cc	Urine flow, cc/min	Plasma vol, cc	Urine flow, cc/min
Salyrgan.						
	LH		ET		V	
0	4634	0.86	5260	0.47	5140	0.53
$\frac{1}{2}$	4486	1.17	5520	.70	5110	.85
1	4386	8.00	5000	.79	4620	1.78
2	4060	11.33	4894	.48	4620	4.58
3			4394	.56		
5			4916	.99	3280	3.78
7			5088	1.29		
9			4826	1.31		
11			4045	1.33		
Aminophyllin.						
	A		R		AO	
0	3440	1.00	6240	1.05	6120	1.17
$\frac{1}{2}$					6488	3.09
1	3713	1.05	6840	2.87	6383	5.14
2	4170	1.70	6720	3.35	6500	5.02
3	3976	1.92	6660	3.14	6697	4.77
5	3537	1.58	6220	2.04	7202	1.80
7	3732		5220	1.70	6278	1.55
9	3649				4913	1.08
11	3683	.16	5580	1.20	4072	.60
Digoxin.						
	A		HB		SC	
0	5070	0.37	3890	0.10	4710	.83
1	5190	.56	4130	4.52	4310	3.84
2	5150	.85	3640	2.24	4237	3.67
3	4970	.91	3682	5.20	4273	1.80
5	5170	.90	3800	2.26	4164	6.08
7	5140	1.33	3790	3.43	3982	4.50
9	4830	1.70	3060	3.81	3947	8.20
11	4446	2.29	2505	3.28	3645	10.00
24	3820	2.25				

injection intravenously of one of the 3 types of diuretic drugs, salyrgan, aminophyllin, and digoxin. The urinary output has also been carefully followed, and the plasma volume correlated with the degree of diuresis. Data from typical experiments are recorded in Table I. It is to be emphasized that under the conditions of our experiments the plasma volume is influenced by fluid loss through the kidneys and fluid mobilization from the tissues. These factors exert an opposite effect, and their relative magnitude determines the blood volume.

When digoxin is injected in a dose of 2 mg there is first noted a slight increase in the plasma volume. This has never been great, and the time of its appearance seems to depend on the attainment

of the desired myocardial effect. As the rate of urinary flow accelerates, this increase disappears and is followed by a reduction, the amount of which is determined by the rate of diuresis.

The fluid eliminated during a rapid salyrgan diuresis seems to come largely from the plasma during the first 8 to 12 hours. After this period, as the rate of diuresis drops, the plasma volume is partially restored. In one of our cases there was a delayed diuresis, in one no diuretic response to the drug, and the plasma volume was slightly increased in each, suggesting that in addition to the usually dominant renal action, the mercurial also exerted an accessory effect on tissue fluid mobilization.⁵

The administration of 0.48 g of aminophyllin intravenously results in relatively large rise in the plasma volume, amounting in various individuals to from 400 to 1200 cc. The first rise coincides with the time of maximal diuresis; the plasma volume drops slightly during the period of rapid urine flow, but the rise continues as the degree of diuresis abates, and persists for approximately 6 hours. After this time the volume drops sharply, presumably due to a return of fluid into the tissues, for the rate of urine production has by then dropped back nearly to the control level. In one experiment the usual rise in plasma volume was preceded 20 minutes after injection by a slight drop. The significance of this finding must be further explored. The striking increase in plasma volume after aminophyllin injection cannot be adequately explained by the available data. The possible mechanisms for tissue fluid mobilization, as well as the concomitant shifts in total circulating plasma protein⁶ are being further investigated.

⁶ Calvin, D. B., *Proc. Am. Physiol. Soc.*, in press.

**Effect of Azosulfamide (Neoprontosil) on Experimental
Welchii Infection in Mice.**

P. MORALES-OTERO AND LUIS M. GONZÁLEZ.

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auspices of Columbia University.*

Bliss and Long¹ report that sulfanilamide has a curative effect in mice infected with *Clostridium welchii*. Carpenter and Barbour² report that oral administration of Neoprontosil prevented death in mice given the toxin of *Clostridium welchii*. We decided to study the effect of Neoprontosil (Winthrop Chemical Co.)* on experimental *welchii* infection in mice.

The strain of *Cl. welchii* used in the following experiments was isolated from a human case of gas gangrene and is similar, in its biological characteristics, to the classical *Cl. welchii* described in textbooks. The culture was passed through Swiss mice before culturing in glucose broth in order to retain its virulence. Its M.L.D. for mice under these conditions was 0.1 cc of a 24-hour glucose broth culture.

Experiment I—Ninety mice, separated into 3 groups of 30 each, were inoculated intramuscularly with ascending doses of a 24-hour glucose broth culture of *Cl. welchii*. The first group was injected with 0.05 cc, the second with .075 cc, and the third with 0.1 cc. Seventy-nine of these mice, or 87.7%, died before 72 hours—20 (66%) in the first group, 29 (96.6%) in the second, and all (100%) in the third.

Ninety additional mice were similarly grouped and inoculated, but besides the corresponding dose of the organisms, each was given 1 cc of Neoprontosil intramuscularly at the time of inoculation. Eighty-five (94.4%) mice died before 72 hours—25 (83.3%) in the first group, and all (100%) in the second and third groups (Table I, Exp. I).

Ten mice were similarly inoculated with 1 cc of Neoprontosil only, and all survived.

¹ Bliss, A. L., and Long, P. H., *J. Am. Med. Assn.*, 1937, **109**, 1524.

² Carpenter, C. M., and Barbour, G. M., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 255.

* The Neoprontosil used was 5% solution, kindly supplied by Mr. Rassow, local representative of the Winthrop Chemical Company.

TABLE I.

Groups studied	No. of mice inoculated	No. of mice that died	% of mice that died
Experiment I.			
Control			
1	30	20	66
2	30	29	96.6
3	30	30	100
Totals	90	79	87.7
Experimental			
1	30	25	83.3
2	30	30	100
3	30	30	100
Totals	90	85	94.3
Experiment II.			
1	30	4	13.3
2	30	7	23.3
3	30	27	90
4	30	27	90
5	30	0	0

In the above experiment, Neoprontosil did not protect mice from *Cl. welchii* infection.

Experiment II—A 24-hour glucose broth culture was centrifuged at high speed. The supernatant was set aside and the sediment was repeatedly washed with saline. After repeated centrifugations and washings, enough saline was added to the cells to restore the original volume of the culture, and 0.1 cc of this suspension was used for the inoculations.

One hundred fifty mice were separated into 5 groups of 30 mice each. Group 1 was inoculated intramuscularly with 0.1 cc of washed cells of *Cl. welchii*. Group 2 was inoculated intramuscularly with 0.1 cc washed cells and 0.1 cc filtrate from the same culture. Group 3 was inoculated with 0.1 cc washed cells and 0.1 cc filtrate, plus 1 cc Neoprontosil, Group 4 with 0.1 cc washed cells plus 1 cc Neoprontosil, and Group 5 with 0.1 cc filtrate plus 1 cc Neoprontosil.

Four mice (13.3%) died in Group 1, 7 (23.3%) died in Group 2, 27 (90%) in Group 3, 27 (90%) in Group 4, and none in Group 5. (Table, Exp. II.) In this case, the injection of Neoprontosil with washed cells of *Cl. welchii* led to the development of the corresponding infection, while the washed cells alone showed little tendency to develop in the tissues after inoculation.

In repeating the above experiment, the dose of Neoprontosil was lowered, using 0.75 cc, 0.50 cc and 0.25 cc, and in every case, when the dose of Neoprontosil was given with washed cells of *Cl. welchii*,

the animal succumbed to infection. On examining the lesions produced, one could observe normal phagocytic activity and numerous organisms within the tissue of the lesions produced. Feinstone, Bliss, Ott and Long³ present evidence indicating that the activity of Neoprontosil depends on its reduction to sulfanilamide *in vivo*.

Gye and Cramer⁴ found that ionizable salts of calcium inoculated together with washed spores of *Cl. welchii* or *Cl. tetani* led to the development of the corresponding infections in their fatal form, while washed spores alone did not lead to death. Fildes⁵⁻⁷ thinks that there must be some definite stimulus to vegetation in the tissues injected by calcium salts, and suggests that this stimulus is probably the result of diminished oxygen tension. He showed further that the injection of solutions of calcium chloride lead to the production of localized areas of oxygen deficiency. We do not know if Neoprontosil in this case acts in a similar way.

Summary—We were unable to protect mice from M.L.D. of *Cl. welchii* by intramuscular injection of Neoprontosil. The intramuscular injection of washed cells of *Clostridium welchii* with Neoprontosil in mice led to the development of a fatal infection.

11518

Blood Progesterone During Sexual Cycle of *Macaca rhesus*; Quantitative Assay.

INÉS L. C. DE ALLENDE.* (Introduced by G. W. Corner.)

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At the suggestion of Professor George W. Corner, the presence of progesterone in the blood during the menstrual cycle of the monkey, *Macaca rhesus*, has been studied and an effort made to

³ Feinstone, W. H., Bliss, E. A., Ott, E., and Long, P. H., *Bull. Johns Hopkins Hospital*, 1938, **62**, 565.

⁴ Gye, W. E., and Cramer, W., *Sixth Sci. Rep. Imp. Cancer Res. Fund*, 1919, pp. 40-57.

⁵ Fildes, P., *Brit. J. Exp. Path.*, 1927, **8**, 387.

⁶ *Ibid.*, 1929, **10**, 151.

⁷ *Ibid.*, 1929, **10**, 197.

* Fellow of the Argentina Association for the Advancement of Sciences. This work was made possible by a grant made to Dr. George W. Corner through the University of Rochester by the John and Mary R. Markel Foundation.

establish the curve of its variation. For this purpose the results recently obtained by McGinty, Anderson and McCullough¹ provide a sensitive qualitative test and within limits (Haskins)² make possible a rough quantitative assay.

Method. Samples of blood were taken every 3 days and the serum extracted with ether. The ether extract was treated with 0.2 N sodium hydroxide and the ether-soluble fraction evaporated and the residue dissolved in peanut oil. A series of dilutions was made in such a way that each 0.1 cc of oil contained the ether-soluble fraction of 1, 2, 3 and 4 cc of normal serum, respectively. The hormone was tested by the method of local intrauterine injections of de Mussio-Fournier, Albrieux, Morato, and Grosso.³ Immature rabbits of an average weight of 710 g were injected daily with 25 I.U. of estrogen (Amniotin) for 6 days. On the 7th day operation was performed under anesthesia, and 2 segments 2 cm long were isolated between ligatures in each uterine horn. Three of these were injected with the dilutions which contained the ether-soluble fraction of the different quantities of normal serum, and the fourth with peanut oil as a control. Autopsies were done at 72 hours after the operation and the segments of the uterus were examined histologically. The degree of endometrial proliferation was evaluated according to the scale of McPhail.⁴

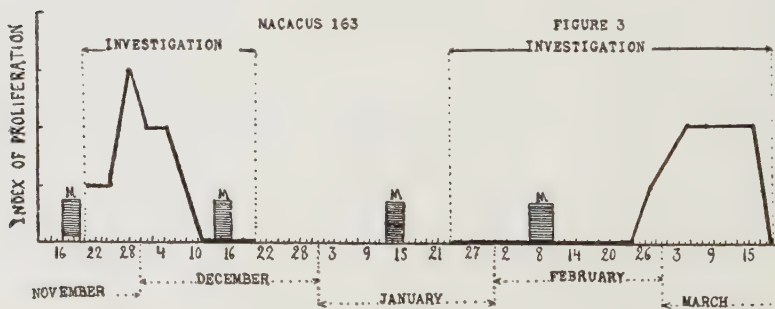
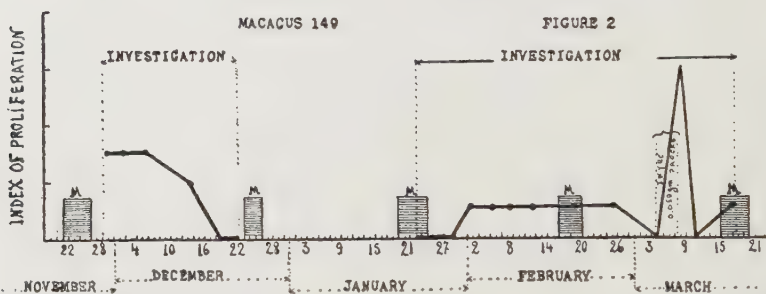
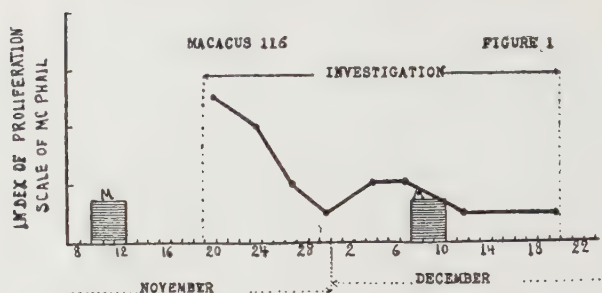
Results. Figs. 1, 2 and 3 show the results obtained in 3 Rhesus monkeys of the same age and weight. The curve represents the variations of the degree of proliferation of the endometrium of the immature rabbit obtained with 1, 2, 3 and 4 cc, respectively, of blood serum, studied every 3 days. The maximum reactions corresponded to the value +++ on the scale of McPhail and the minimum measured between 0 and +. In 60% of those cases in which the serum extract gave a positive reaction in the segments in which it was injected, the segment injected with peanut oil also showed a positive reaction between ++ to +++ and +, probably because the hormone circulating in the blood stream is concentrated in the peanut oil. For this reason, in the second series the use of peanut oil as a control was abandoned. In the first study, made during the November-

¹ McGinty, D. A., Anderson, L. P., and McCullough, M. B., *Endocrinology*, 1939, **24**, 829.

² Haskins, Arthur L., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 624.

³ Mussio Fournier, J. C., Albrieux, A. S., Morato, J., and Grosso O., *Bull. de l'Acad. de Med. Paris*, 1938, **120**, 273; *Rev. de Obstet. e Ginec. de Sao Paulo*, 1938, **3**, 203; *Endocrinology*, 1939, **24**, 515.

⁴ McPhail, M. K., *J. Physiol.*, 1935, **83**, 145.



Curves of progesterone in the blood during the sexual cycle of *Macaca rhesus*.

December cycle, the curve shows in monkeys 116 and 163 (Fig. 1 and 3) a maximum reaction of +++ and ++ to +++, respectively, on the 10th and 11th day of the cycle, while in monkey 149 (Fig. 2) it is maintained at the same level (+ to ++) during this first half of the cycle. In the 3 animals the curve drops gradually in the second half of the intermenstrual period; but while in 2 of them (Fig. 2 and 3) it becomes negative 3 and 5 days before the next menstruation, in monkey 116 (Fig. 1) it reaches a proliferative reaction equal to + the day before the catamenial flow.

The great similarity of results in this first cycle was not observed when the investigation was repeated in animals 163 and 149.

Monkey 163, which showed all negative reactions in the second half of the January-February cycle (Fig. 3) maintained this negativity, during the first and part of that which would have constituted the second part of the February-March cycle, which was incomplete due to the failure of menstruation in the latter month. If we assume that menstruation would have occurred between the third and the seventh of March, the maximum reaction (++) was observed precisely in this period and the curve maintains this maximum during what would have been the first half of the following cycle, thus being in accord with the first study made. In monkey 149 (Fig. 2) weak reactions were obtained at the end of the first and during the second half of the January-February cycle and moderate reactions in the first part of the February-March cycle.

In order to control the procedure used we injected this animal with 0.010 g of progesterone daily for 5 days. Blood taken on the sixth day gave a +++ reaction of proliferation with 2 cc of serum, successive samples being negative.

Quantitative Assay. If we use the figures found by McGinty to evaluate the uterine proliferative reactions we should be able, by trying measured doses, to calculate indirectly the quantity of progesterone existing in the blood at any given moment. If as stated by this author, ++ to +++ reactions in the uterus of the rabbit are produced by doses of progesterone which vary from 5 to 0.5 γ , when we obtain in our case the same reaction with 2 cc of serum, we may deduce that each cubic centimeter of blood serum contained, at the moment it was taken, between 2.5 and 0.25 γ of progesterone.

Conclusions. 1. The presence of progesterone was demonstrated in the blood of the normal *Macaca rhesus*. 2. The hormone in 1, 2, 3 and 4 cc of blood serum was studied every 3 days by means of local intrauterine injection in immature rabbits and the degrees of progestational reaction of the endometrium measured according to the scale of McPhail. 3. With this procedure it was possible to obtain the curve of its variations during a complete menstrual cycle in 3 Rhesus monkeys of the same age and weight. 4. In 2 of the animals the curve shows its maximum (++ to +++ reaction) on the 10th and 11th day of the menstrual cycle respectively, while in the third it was maintained at a constant level (+ to ++ reaction) during the first half of the cycle. In the 3 monkeys the curve gradually fell during the second half of the menstrual cycle. 5. Studies repeated in 2 of the first animals, during 2 complete cycles, did not give such clear and definite results, perhaps because

of lesser sensitivity of the rabbits to the same small quantities of progesterone which were found in the first cycle, rather than to a diminution of the circulating hormone. 6. By calculation from the figures obtained by McGinty, we arrive at the conclusion that during the menstrual cycle of one of the animals studied, the amount of progesterone in 1 cc of blood serum at any given moment varied between a maximum of 0.25 to 2.5 γ and a minimum of 0.06 to 0.12 γ .

11519

Blood Volume in Experimental Hypertension Following Subtotal Nephrectomy. Effect of Posterior Pituitary Lobectomy.

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From the Robinette Foundation, the George S. Cox Medical Research Institute, and the Medical Clinic of the Hospital of the University of Pennsylvania.

Chanutin and Ferris¹ described the development of vascular hypertension in rats after a 2-stage operation in which two-thirds of one kidney and, later, all of the remaining opposite kidney were removed. McQueen-Williams, quoted by Winternitz,² noted that hypertrophy of the fragment of kidney remaining did not occur in such an animal if the pituitary was removed. It was not stated whether the hypophysectomy had any effect upon the development of the vascular hypertension. The following studies were undertaken to determine whether there was any blood volume change associated with an inadequate amount of renal substance, and what relationship the pituitary, especially the posterior lobe, might bear to such change and to the blood pressure.

Procedure 1. A series of rats were subjected to the procedure described by Chanutin and Ferris; 16 rats survived. Seven to 10 days later blood pressure was measured by the indirect method of Griffith³ and blood volume by the method of Griffith and Campbell.⁴

* Atwater Kent Fellow in Medicine.

¹ Chanutin, A., and Ferris, E. B., Jr., *Arch. Int. Med.*, 1932, **49**, 767.

² McQueen-Williams, M., unpublished. Quoted by Winternitz, M. C., Thomas, R. M., and LeCompte, P. M., *The Biology of Arteriosclerosis*, C. C. Thomas, 1938.

³ Griffith, J. Q., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1934, **32**, 394.

⁴ Griffith, J. Q., Jr., and Campbell, R., *Proc. Soc. Exp. Biol. and Med.*, 1937, **36**, 38.

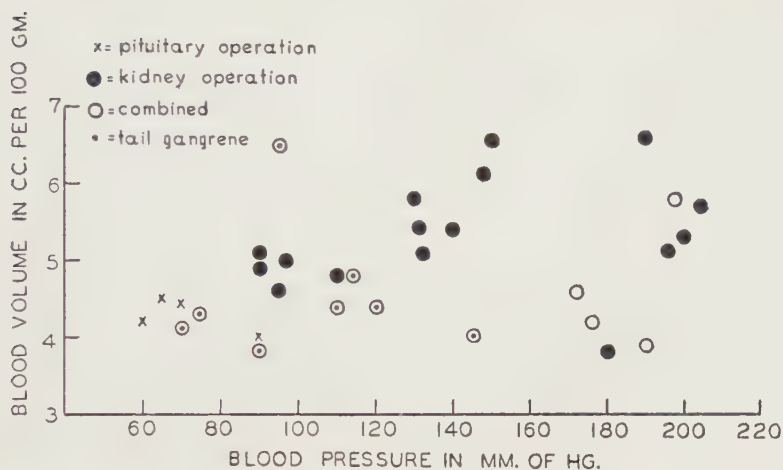


FIG. 1.

Blood pressure of the normal rats, under nembutal anesthesia, will not exceed 140 mm of mercury when measured by this method, and less than 5% of the animals will show pressures in excess of 120. Blood volume, expressed in cc per 100 g body weight ranges from 4.0 to 5.0 cc in normal animals weighing over 160 g while the upper limit in animals weighing less than 160 g is 5.3. All animals used in this study weighed well over 160 g.

It is seen in Fig. 1 that in half of the 16 animals, represented by solid dots, the blood pressure was 140 or over. In all rats with blood pressures of 140 or higher the blood volume was 5.1 or more except in one case, a female with a blood pressure of 180 and a blood volume of only 3.8. This animal may have been pregnant and, if so, should not be included in the series. Some elevation of blood volume occurred in 3 animals with blood pressures over 130, while the remaining animals with definitely normal blood pressures tended to have blood volumes within the normal range.

Procedure 2. The posterior lobe and half the anterior lobe of the pituitary were resected in 4 animals by the usual parapharyngeal route. All promptly developed typical diabetes insipidus. Blood pressure and blood volume measurements, made 7 to 10 days later, are charted as crosses in Fig. 1. All were normal.

Procedure 3. Fourteen animals survived the combined pituitary and kidney operations as described in procedures 1 and 2. The operations were performed in 3 stages a week apart, the usual order being subtotal nephrectomy, pituitary operation, nephrectomy. The first blood pressure measurement was made one week after the last

operation. Only 2 of the 14 animals were hypertensive. This measurement was repeated one week later, when 7 out of 14 were found to be hypertensive. Thus the appearance of the hypertension was somewhat delayed. At this time blood volume was measured in 12 animals (one died and in one there was a technical error in the intravenous injection of the dye). The results are shown in Fig. 1 as open circles. Blood volume was increased in only 2 animals, one of which was hypertensive while one was not. Blood volume was normal in 4 animals with hypertension.

As an unexpected finding, certain animals undergoing the combined operations as outlined in procedure 3 developed gangrene of the tail, which may be described as moist in contrast with the dry gangrene seen after ergotamine poisoning. This occurred in 8 of the 13 surviving animals, 12 of which are charted in Fig. 1. Rats with gangrene are indicated by dots placed centrally in the open circles. The 13th animal had a blood pressure of 163, no tail gangrene, but blood volume determination failed and, therefore, it is not charted. It is apparent that there was a tendency for animals developing hypertension to escape the gangrene.

Comment. While the number of animals is too small to permit absolute conclusions, certain tendencies are obvious. The increase in blood pressure occurring in rats with only about half of one kidney remaining is associated, as a rule, with increased blood volume. In the absence of the posterior lobe of the pituitary, such elevation in blood volume does not occur, but the vascular hypertension is unaffected. This may mean that the increased blood volume is maintained through the mediation of the posterior lobe of the pituitary, or, more likely, that in the presence of a marked continuous diuresis an increased blood volume is more difficult to maintain. The gangrene of the tail is difficult to explain. It has never occurred in several hundred cases where either the kidney or the pituitary operations were performed alone. It would appear that without a vascular hypertension the animal is unable adequately to maintain its peripheral circulation under the conditions detailed in procedure 3.

Summary. Experimental hypertension may develop in the partially nephrectomized rat in either the presence or absence of the posterior pituitary. In the presence of the posterior pituitary 7 of 8 hypertensive rats showed an increase in blood volume whereas in the absence of the posterior pituitary 4 of 5 hypertensive rats showed a normal blood volume.

11520

Axon Branching After Nerve Regeneration.*

W. G. WATROUS. (Introduced by J. M. D. Olmsted.)

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Berkeley, California.*

Langley and Anderson¹ proved by physiological methods that regenerating axons often branch; Kilvington² employed both physiological and histological methods to demonstrate the multiplication of axons in the regenerating segment of a peripheral nerve. Bender and Fulton³ showed functional disorders after regeneration of the oculomotor nerve in the chimpanzee and attributed them to fiber splitting and aberrancy. There is complete unanimity regarding the presence of branching axons during regeneration, and in fact this response has been used as a method to make up for the deficit of axons in cases of anterior poliomyelitis (Feiss,⁴ Dogliotti,⁵ Aird and Naffziger,⁶ and others). To our knowledge, however, there have been no experiments reported which directly record those muscular contractions which fiber branching makes possible through the axon-reflex.

After section of the peroneal and popliteal branches of the sciatic nerve at the knee in the cat and dog and regeneration has well commenced, an opportunity is afforded to show the axon-reflex contraction in both the anterior and the posterior tibial muscles. Stimulation of the posterior tibial nerve (a mixed nerve) at the heel will cause a contraction of the gastrocnemius and stimulation of the superficial peroneal nerve (sensory only) at the ankle will provoke a contraction in the tibialis anticus even after cutting the sciatic in the thigh (Fig. 1). With the use of a strong tetanizing current, the curves are similar in form to those produced by stimulating the appropriate motor branch of the sciatic nerve with a like current. Progressive downward section of the sciatic nerve to a point just above the neuroma does not affect the contraction in the least, but

* This investigation has been supported by a grant from the Research Board of the University of California.

¹ Langley, J. N., and Anderson, H. K., *J. Physiol.*, 1902, **29**, iii.

² Kilvington, B., *Brit. Med. J.*, 1905, **1**, 935.

³ Bender, M. B., and Fulton, J. F., *J. Neurophysiol.*, 1938, **1**, 144.

⁴ Feiss, H. O., *Bost. Med. and Sci. J.*, 1911, **164**, 667.

⁵ Dogliotti, A. M., *J. de Chir.*, 1935, **45**, 30.

⁶ Aird, R., and Naffziger, H. C., *Arch. Surg.*, 1939, **38**, 906.

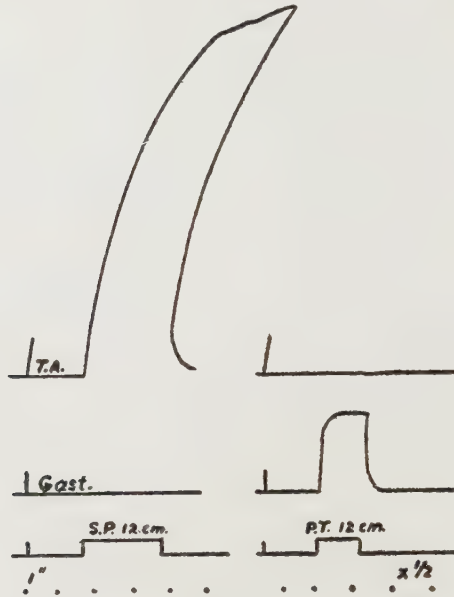


FIG. 1.

Tracing of an axon-reflex contraction of the tibialis anticus on stimulation of the superficial peroneal nerve, and of the gastrocnemius on stimulation of the posterior tibial nerve, after cutting the sciatic in the thigh.

section of the sciatic nerve just below the neuroma immediately and permanently abolishes the axon-reflex contraction. No histological counts were made, but an idea of the degree of fiber-splitting may be gained from the fact that such an axon-reflex contraction may be as much as three-fourths the height of the contraction caused by maximal motor nerve stimulation.

The fiber branching undoubtedly contributes to the incoordination which follows nerve section and nerve regeneration in the limb, as previously shown by Bender and Fulton³ for the eye, but since the peroneal nerve innervates exclusively flexors and the popliteal nerve innervates mainly extensors, this factor is minimized. The axon-reflexes furthermore persist and are not eliminated by a process of atrophy as suggested by Langley⁷ for aberrant fibers, since they are found in full force even after eighteen months.

Summary. Confirmation of axon branching as a result of nerve regeneration has been obtained by physiological methods. The axon-reflex contraction made possible through such branching possesses all the characteristics of a muscular contraction evoked by direct electrical stimulation of the motor nerves concerned.

⁷ Langley, J. N., *J. Physiol.*, 1897, **22**, 215.

11521

Generalized Edema in Chicks Prevented by d, l-Alpha Tocopherol.*

H. R. BIRD AND THOS. G. CULTON. (Introduced by T. C. Byerly.)

*From the Department of Poultry Husbandry of the University of Maryland,
College Park, Maryland.*

The experiments here reported were designed to study the nutritive completeness of different samples of dried skimmilk for chicks. A basal ration was developed in which all the protein is supplied by dried skimmilk and the known deficiencies of dried skimmilk are corrected by the addition of small amounts of various supplements. This ration, referred to as Ration 3, has the following composition: dried skimmilk 54%, dextrinized starch 44%, ground limestone 1%, and NaCl 1%, plus 0.12% of ferric citrate, 0.012% of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ and 0.0012% of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Cod liver oil is administered by pipette thrice weekly. Chicks consume this diet readily but grow at a somewhat subnormal rate.

When certain samples of dried skimmilk are fed as a part of this ration, a severe generalized edema develops in a large percentage of the chicks. The first manifestation of this condition is extreme subcutaneous edema in some chicks as early as 3 weeks of age, and sufficient in extent to be readily detected without handling the chicks. Many chicks develop a characteristic straddling stance, the legs being forced apart by the great accumulation of fluid under the skin of the ventral body surface. The subcutaneous edema may also extend to the neck and back of the head. No recoveries have been observed; all birds so affected have died within 2 or 3 weeks. Death is frequently preceded by stupor, seldom lasting longer than 10 to 12 hours. Labored breathing is frequently observed during this period.

A considerable proportion of the chicks die between the ages of 3 and 9 weeks without manifesting any subcutaneous edema. The most consistent post mortem finding in such chicks, as well as in those with subcutaneous edema, has been extreme distention of the heart and pericardium, the latter being filled with exudate. Other common post mortem findings have been ascites, edema of the brain and lungs, and coronary and intestinal hyperemia. In one case, in excess of 50 cc of ascitic fluid were removed from a 600 g bird.

* Supported in part by a grant from the American Dry Milk Institute, Inc.
Scientific Contribution No. 514, Maryland Agricultural Experiment Station.

TABLE I.
Mortality and Incidence of Edema in Chicks Fed Ration 3 with and without Supplements.

Exp. No.	Skim milk	Supplement	No. of chicks	% edema		% mortality	
				5 wk	8 wk	5 wk	8 wk
2	S*	0	13	62		62	
	W*	0	16	19		31	
3	S	0	30	37	53	50	73
	W	0	30	57	77	67	90
6	S	0	33	30	42	51	93
	W	0	32	31	53	59	94
	S	3% dehydrated grass	32	0	0	9	25
	W	3% " "	32	0	0	3	9
	S	6% yeast	32	25	50	41	72
	W	6% " "	31	26	46	46	89

* S = Summer; W = Winter.

The first proof that the edema was the result of dietary deficiency was afforded by an experiment in which dehydrated cereal grass† was fed as a supplement to the basal ration. The results of this and other experiments are summarized in Table I. Samples of dried skim milk prepared in summer and in winter were fed, but no difference in response was observed. The figures in the table establish the protective effect of grass and the lack of protective effect of yeast.

A trial of the effectiveness of d,l-alpha tocopherol was suggested by the report of Dam and Glavind¹ on its effectiveness in preventing the "alimentary exudative diathesis" described by them. Synthetic d,l-alpha tocopherol‡ was dissolved and suitably diluted with cod liver oil and administered thrice weekly to a group of 10 chicks in doses of such size as to approximate 7.5 μ g per gram live weight per day. Dosing was begun when the chicks were 4 days of age and continued to the age of 32 days at which time the supply of alpha tocopherol was exhausted. The negative control group consisted of 15 chicks. The results of this experiment are shown graphically in Fig. 1 which shows the cumulative incidence of edema and the cumulative mortality associated with edema. The first case of edema did not appear in the treated group until the chicks were 9 weeks old, 4½ weeks after the administration of the last dose of alpha

† Supplied by the American Butter Company, Kansas City, Mo., through the courtesy of Dr. W. R. Graham, Jr.

‡ Supplied by Merck and Co., Inc., Rahway, N. J., through the courtesy of Dr. G. W. Lewis.

¹ Dam, H., and Glavind, J., *Nature*, 1939, **143**, 810.

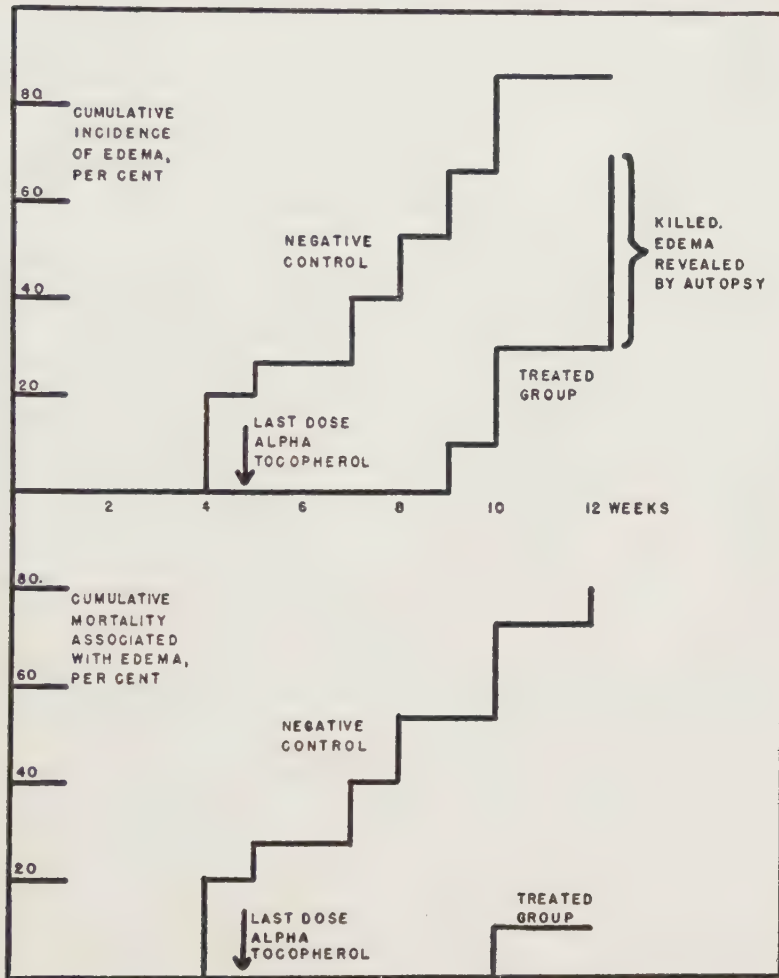


FIG. 1.

Effect of administration of d,l-Alpha Tocopherol on incidence of edema and mortality associated with edema in chicks fed Ration 3.

tocopherol. At this time 10 of the 15 untreated birds had become edematous. The first death associated with edema in the treated group occurred at 10 weeks of age, at which time 11 of the 15 untreated chicks had died with edema. These results would seem to show conclusively that this disease is a manifestation of a deficiency of d,l-alpha tocopherol.

Comparison of the symptoms described here with those described in detail by Dam and Glavind² would seem to indicate that the differ-

² Dam, H., and Glavind, J., *Skand. Arch. für Physiol.*, 1939, **82**, 299.

ences are differences of degree. The Danish workers report exudation in subcutaneous tissues and, rarely, in the cavum peritoneum; and they report, further, that the disease as observed by them terminates frequently in recovery and occasionally in death. From this standpoint as well as from the standpoint of ease of preparation, the ration used in these studies would appear to offer greater possibilities for vitamin E assay than the ration used by Dam and Glavind, who have suggested that an assay method might be developed on the basis of their experiments. Further experiments, designed to explore these possibilities, are in progress.

It is of interest to point out that localized edema is consistent and conspicuous among the histopathological changes in the brains of encephalomalacic chicks, as reported by Pappenheimer *et al.*,³ since Dam *et al.*⁴ have found this disease also to be preventable by d,l-alpha tocopherol. It should be pointed out also that Pappenheimer *et al.* reported a very low incidence of subcutaneous edema in encephalomalacic chicks observed by them. In these experiments edema of the brain was frequently noted, but in no case were the symptoms of encephalomalacia observed in the living edematous birds. Two cases of encephalomalacia were observed among the groups in which generalized edema was prevented by feeding dehydrated grass. This is in agreement with Dam's statement that encephalomalacia occurs on a higher intake of vitamin E than does exudative diathesis.

Dam and Glavind² have pointed out that the edema-producing diets used by them were very low in fat, and have discussed the relationship of this fact to the finding of Pappenheimer *et al.*³ that the incidence of encephalomalacia was increased by increasing the fat content of the diet. They postulate further that vitamin E may act in two different ways against encephalomalacia and exudative diathesis respectively. It may be noted that the edema-producing diet described in this paper is also low in fat although none of the constituents were subjected to extraction to lower their fat content.

The results of the experiments with alpha tocopherol are of some interest from the standpoint of method of administration since it has been reported by Madsen *et al.*⁵ and by Morris⁶ that cod liver oil favors the development of muscular dystrophy in rabbits and

³ Pappenheimer, A. M., Goettsch, M., and Jungherr, E., *Conn. Agr. Exp. Sta. Bul.*, 229, 1939.

⁴ Dam, H., Glavind, J., Bernth, O., and Hagens, E., *Nature*, 1938, **142**, 1157.

⁵ Madsen, L. L., McCay, C. M., and Maynard, L. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1933, **30**, 1434.

⁶ Morris, S. G., *Science*, 1939, **90**, 424.

guinea pigs and hence may be assumed to exert a destructive effect on vitamin E. Such destruction was not evidenced under the conditions of this experiment, and administration of alpha tocopherol in cod liver oil appears to be an entirely practical procedure in experiments of this kind. Nondestearinated U.S.P. cod liver oil was used, and 100 mg of alpha tocopherol were dissolved and diluted in this oil at one time. Such an amount was sufficient to last for 7 to 10 days. It was kept in the refrigerator when not in use.

Summary. A large percentage of chicks fed a laboratory diet of dried skim milk, dextrinized corn starch, cod liver oil, and mineral salts develop a generalized edema and die. The most consistent post mortem finding is edema of the heart and pericardium. This disease can be prevented by administration in cod liver oil of synthetic d,l-alpha tocopherol. The disease has not been observed in any chicks fed practical rations.

11522

**Effect of Amino Acids, of Vitamin B Complex and Other
Compounds on Respiration of Bakers' Yeast.**

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EILERT, S.S.J.* (Introduced by S. Tashiro.)

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In our laboratories a considerable amount of work has been done on the effects of various fractions from yeast and other sources on the respiration and proliferation of yeast and tissues. In this connection experiments have been performed with pure substances some of which may be present in these preparations. The results of these experiments are the subject of the present paper.

Although we have used pure cultures of yeast in some of our respiration work (pure cultures are always used in proliferation studies), it has been desirable to have a readily available and reasonably constant source of yeast in quantity. We have found that Fleischmann's bakers' yeast answers these requirements fairly well. (Anheuser-Busch bakers' yeast has also been used with equal satisfaction in later work, but the experiments herein reported deal with

* Assistance in some experiments was given by Vincent Sacksteder.

Fleischmann's.) To insure sufficient uniformity we always use the yeast within 3 days after the "cutting" date.

All determinations were made by the direct Warburg method, using the technic previously described.^{1,2} Fleischmann's bakers' yeast from the center of a fresh 1-lb cake was washed 2 times by centrifugation with the suspending medium (Ringer-phosphate-glucose; 0.02% glucose; pH 7.3). The yeast was then made up with fresh medium to give a count of 250 as determined by a photo-electric densitometer. A count of 1 equals 250,000 cells per cc, or 0.064 mg (wet weight) of yeast per cc. One cc of the yeast suspension (containing an average of 3.9 mg dry weight of yeast), the desired amount of test solution and sufficient Ringer-phosphate-glucose to give a volume of 3.1 cc were placed in the outer well of the manometer flask and 0.2 cc of N KOH was placed in the inner well (trial showed the use of KOH papers to be unnecessary). After an equilibrium period of 15 minutes, the manometers were shaken for a 25-minute respiration period, in air, at 37.5°C. Under these conditions the control respirations usually ran between 40 and 60 mm³ corresponding to a $Q_{O_2}^{air}$ of about 25 to 37.

The substances tested were usually dissolved, or in some cases suspended, in Ringer-phosphate-glucose; a few of the less soluble materials were dissolved in distilled water. After adjustment of pH to 7.3 with sodium hydroxide or hydrochloric acid, the concentration was such that no more than 1 cc of the solution was added to the flasks. Certain of the substances, normally insoluble, were thus dissolved as the sodium salts.

Variations of $\pm 5-7\%$ are within experimental error. Only stimulations of 10% or greater are regarded as being significant. All data reported are the result of 2 to 6 determinations at each of not fewer than 4 concentration values within the limits given.

Of the amino acids examined in concentration ranges of 0.069 to 1.0 mg per cc, the following were inactive: d-alanine, arginine, cystine, glutamic acid, glycine, l-histidine, dl- β -phenylalanine, l-proline, and l-tryptophane. The following, in different experiments, varied from inactive to slightly active (10-15% stimulation): dl-alanine, l-alanine, β -alanine, l-aspartic acid, l-leucine and tyrosine.

In another series of experiments several members of the vitamin B complex were examined.[†] Of these, nicotinic acid (0.077-0.77

¹ Cook, E. S., Kreke, C. W., and Nutini, L. G., *Studies Inst. Divi Thomae*, 1938, **2**, 23; Cook, E. S., Hart, M. J., and Joly, R. S., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 169.

² Cook, E. S., and Morgan, M. N., *Biochem. J.*, 1940, **34**, 15.

TABLE I.
Effect of Thiamin Hydrochloride on Respiration of Bakers' Yeast.

Cone., mg/cc	% stimulation
.008	8
.017	6
.042	12
.083	26
.166	26
.420	34
.830	0

mg/cc) and riboflavin (0.0097-0.97 mg/cc) were inactive. Vitamin B₆ (0.01-0.98 mg/cc) was generally inactive although occasional stimulation of the order of 10% was found with some batches of yeast. Thiamin hydrochloride showed an activity dependent upon the concentration, there being an optimum concentration range as shown by the results of a typical experiment in Table I. Of the 2 components of the thiamin molecule, 4-methyl-5- β -hydroxyethyl thiazole showed no significant activity in concentrations of 0.012 to 1.15 mg per cc. 2-Methyl-4-amino-5-ethoxymethyl pyrimidine was inactive between 1.05 and 0.11 mg per cc; slight activity (10-13%) was found in concentrations down to 0.01 mg per cc. However, an equimolecular mixture of the 2 showed the same activity as thiamin in total concentration ranges of 0.05 to 0.50 mg per cc (*e.g.*, at 0.1 mg/cc, thiamin gave 29% and the mixture 30% stimulation).

The following substances were also inactive in stimulating respiration: adenosine phosphate (Hoffmann-LaRoche) (0.076-0.76 mg/cc), yeast nucleic acid (0.08-0.8 mg/cc), thymus nucleic acid (0.08-0.8 mg/cc), inositol (0.097-0.97 mg/cc) and hippuric acid (0.094-0.94 mg/cc).

Insulin (Iletin, Lilly) was inactive in a concentration of 0.016 mg (or 0.13 unit) per cc but caused 18% stimulation in 10 times this concentration. Concentrations of 0.8 mg (6.5 units) and 1.6 mg (13 units) per cc gave, respectively, 240% and 330% stimulation.

From the results it is seen that most of the amino acids, under the conditions used, were inactive or of a very low order of activity. It will be recalled that l-leucine³ and β -alanine^{3,4} (by itself or com-

† We wish to thank Drs. R. T. Major and J. M. Carlisle of Merck and Co. for gifts of synthetic thiamin, 2-methyl-4-amino-5-ethoxymethyl pyrimidine, 4-methyl-5- β -hydroxyethyl thiazole, riboflavin and vitamin B₆.

³ Miller, W. L., *Trans. Roy. Soc. Can.*, III, 1936, **30**, 99.

⁴ Williams, R. J., and Rohrmann, E., *J. Am. Chem. Soc.*, 1936, **58**, 695.

bined in pantothenic acid^{5,6}) have been identified as bios components for various yeasts. Pratt and Williams⁷ found that, while both pantothenic acid and β -alanine increased the respiration of deficient Gebrüder Mayer yeast, pantothenic acid was ineffective on Fleischmann's cake yeast, presumably owing to an adequate supply of pantothenic acid in the latter yeast. This checks our observations on the usual inactivity of β -alanine. The relative ineffectiveness of the amino acids contrasts markedly with the stimulating effects of non-toxic concentrations of saturated fatty acids on bakers' yeast; these effects appear to be due to the ability of the yeast to oxidize the fatty acids.²

Among the other substances examined, thiamin, vitamin B₆, and inositol may also act as bios components for certain strains of yeast. Of these, only thiamin was definitely effective in stimulating the respiration of bakers' yeast. Williams⁷ found similar results with both Gebrüder Mayer and Fleischmann's yeasts. It is of interest that, while the 2 components of the thiamin molecule are essentially inactive by themselves (the pyrimidine portion showing only very slight activity), an equimolecular mixture is as effective as the intact molecule. This suggests an ability of the yeast to combine the thiazole and pyrimidine moieties into the whole molecule which would seem to be essential for an increase in respiration. In contrast, Schultz, Atkin and Frey⁸ found the pyrimidine portion to be equally as effective as thiamin in stimulating fermentation by bakers' yeast, the thiazole portion being ineffective.

The inactivity of yeast nucleic acid checks the observation of Pourbaix⁹ who found that sodium nucleinate has no effect on the normal respiration of yeast; it will, however, restore to normal respiration which has been depressed by styryl 430.

The very marked effect of insulin in increasing the oxygen uptake of yeast is particularly interesting in view of the reports that it does not accelerate the fermentation of glucose by yeast.¹⁰ These experiments are being extended in our laboratories.

⁵ Williams, R. J., and Saunders, D. H., *Biochem. J.*, 1934, **28**, 1886.

⁶ Rainbow, C., *J. Institute Brewing*, 1939, **45**, 533; Rainbow, C., and Bishop, L. R., *ibid.*, 1939, **45**, 593.

⁷ Pratt, E. F., and Williams, R. J., *J. Gen. Physiol.*, 1939, **22**, 637.

⁸ Schultz, A. S., Atkin, L., and Frey, C. N., *J. Am. Chem. Soc.*, 1937, **50**, 2457.

⁹ Pourbaix, Y., *Compt. rend. soc. biol.*, 1939, **131**, 1306.

¹⁰ Fürth, O., *Biochem. Z.*, 1923, **150**, 265; Lauffberger, V., *Z. ges. exp. Med.*, 1924, **42**, 570; Travell, J. G., and Behre, J. A., *Proc. Soc. Exp. Biol. and Med.*, 1923-4, **21**, 478; Euler, H. von, and Myrback, K., *Z. physiol. chem.*, 1925, **150**, 1.

Obviously, as with growth factors, the effectiveness of various substances on the respiration of yeast will depend upon the strain of yeast, the medium, and the general technique. The present results are of value in checking against the activity of various fractions from yeast and animal tissues which we are assaying by means of the technique used in this paper. Under different conditions certain of the apparently inactive materials may assume importance when they become limiting substances. Suggestions of this are seen in the case of several amino acids and vitamin B₆.

Summary. A number of amino acids, members of the vitamin B complex, and miscellaneous substances have been examined for their effects on the respiration of Fleischmann's bakers' yeast. Most of the substances are inactive or only slightly active, but thiamin, or a mixture of its pyrimidine and thiazole components, and insulin have marked activity.

11523 P

Quantitative Studies of Cell Types in Rat Hypophysis Following Administration of Antigonadotropic Serum.*

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Several reports in the literature have shown that treatment of animals with antigonadotropic serum causes a condition in the pituitary gland analogous to that found in castrated animals. Severinghaus and Thompson^{1, 2} have described cytological changes in the hypophyses of dogs injected with antihormones. In these animals there was an increase in the basophile cells and a corresponding decrease in the chromophobes which was associated with an atrophy of the gonads, thyroids, and adrenals. Physiological effects have been demonstrated in rats by Meyer and Kupperman³ who

* Aided in part by a grant from the Wisconsin Alumni Research Foundation and by assistance furnished by the personnel of W.P.A. Official Project No. 65-1-53-2349.

¹ Severinghaus, A. E., and Thompson, K. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **40**, 627.

² Severinghaus, A. E., and Thompson, K. W., *Am. J. Path.*, 1939, **15**, 391.

³ Meyer, R. K., and Kupperman, H. S., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **42**, 285.

found that hypersecretion of the pituitary gonadotropic hormone followed treatment with antigonadotropic serum. This effect was determined by precocious development of the ovaries after discontinuing the injections and by ovarian hypertrophy in female rats in parabiosis with pretreated male or female littermates. The work to be reported here corroborates these findings and describes the changes in the number of cells of each of the types in the pituitary gland of female rats following a short period of antigonadotropic serum treatment.

Experimental procedure. Twenty-one female rats were injected subcutaneously from the 10th to the 20th day of life with 0.5 cc per day, or a total dose of 5 cc of antigonadotropic serum obtained from rabbits which had been injected daily with an aqueous extract of whole dried pituitary gland of sheep for a period of 2 months or longer. The serum was shown to be capable of inhibiting the gonadotropic effects of sheep, rat, and human pituitaries, and of pregnant mare serum and prolactin. Three rats were killed on the 1st, 3rd, 5th, 7th, 9th, 12th, and 15th day after discontinuing the treatment. Littermate control rats were autopsied at the same age. Pituitary and ovarian weights with the qualitative ovarian response were noted for both the experimental and control animals. The pituitary glands were serially sectioned at 6 microns after fixation in Bouin's fluid and stained with a modification of Mallory's trichrome stain (Rasmussen⁴). Three horizontal sections from equidistant levels in the gland were studied in each animal. In each of these sections all the cells in every tenth field were differentially counted. An average of 2800 cells was counted in each gland.

Results and discussion. The changes in the percentages of cell

TABLE I.
Ovarian Weights and Percentage of Pituitary Cell Types After Antigonadotropic Treatment.

Group*	Day killed	Avg ovarian wt (mg)	Avg % of pituitary cell types		
			Basophiles	Acidophiles	Chromophobes
1	20	5.0	31.9	17.3	50.8
2	22	7.5	27.4	17.2	53.9
3	24	20.0	20.8	18.0	61.4
4	26	20.0	18.3	18.9	62.8
5	28	20.7	14.8	18.3	66.9
6	31	30.3	12.4	20.3	67.2
7	34	59.7	9.3	19.4	71.3

*Each group was comprised of 3 rats which were injected from the 10th to the 20th day of life.

⁴ Rasmussen, A. T., *Am. J. Anat.*, 1930, **46**, 461.

types in the pituitary gland and the resulting ovarian weights which follow cessation of the antigonadotropic serum treatment are recorded in Table I.

In rats treated for 10 days with antigonadotropic serum and killed on the day following the last injection, the pituitary glands exhibited a picture of extreme basophilism; 31.9% of all the cells were basophiles, in contrast to 5-10% found in normal animals of the same age. The average percentage of chromophobes in these treated animals was 50, showing a definite decrease from the normal level of 70% found in littermate controls. Since no perceptible variation could be detected in the percentage of acidophiles from the normal level during or after treatment, the increase in the number of basophiles appeared to account for the corresponding decrease in chromophobes. The basophilism produced by the injection of antigonadotropic serum appeared to be the same as that found in castrated rats.

Animals killed at later intervals after cessation of treatment showed a progressive decrease in the percentage of basophiles and a proportionate increase in the percentage of chromophobes until about 15 days after the injections were discontinued. At this time the normal ratio of cell types again prevailed, indicating a change of basophiles to chromophobes. Accompanying the decrease in the basophilic elements of the pituitary gland there was a rapid ovarian growth which approached the normal level between the 3rd and 5th days and continued to increase far beyond normal so that by the 15th day after cessation of treatment an average ovarian weight of 59.7 mg had been attained. The average ovarian weight of littermate controls at this age was 17 mg.

Summary. Treatment with antigonadotropic serum resulted in extreme basophilism of the hypophysis of young female rats. When the treatment was discontinued the percentage of basophiles gradually returned to normal with a corresponding increase in the percentage of chromophobes, and marked increase in the size of the ovaries over that of littermate controls.

11524 P

Use of Sodium Hexametaphosphate as an Anticoagulant.

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Sodium hexametaphosphate has recently become established as an effective agent in reducing calcium ion concentration. Most of the research work on the compound has been done in the field of industrial chemistry where it has demonstrated its use as an outstanding water softener.

In this report we are concerned with the mechanism by which this compound reduces calcium ion concentration. The consensus seems to favor the formula $(\text{NaPO}_3)_6$ or $\text{Na}_6\text{P}_6\text{O}_{18}$. This compound can react with calcium ion to form $\text{Na}_2\text{Ca}_2\text{P}_6\text{O}_{18}$ thus leaving the calcium in the form of a complex since the compound ionizes into 2 sodium ions and the negatively charged $\text{Ca}_2\text{P}_6\text{O}_{18}$ ion. The dissociation of this complex ion to yield calcium ion is so slight that the addition of sodium hexametaphosphate depresses the calcium ion to such an extent that it will dissolve the most insoluble calcium salts such as the carbonate, the oxalate, and the phosphate. At present it is impossible to express mathematically the equilibrium constant for the dissociation of the complex ion but indications are that its effective value approaches 10^{-15} .

Comparatively little work has been done on the biochemical and physiological properties of the compound. This paper represents the first report of results of work in progress in our laboratories on the application of properties of sodium hexametaphosphate to the fields of biology and medicine.

The effect of this compound on the clotting of blood was tested by the following method. A stock solution of 20% sodium hexametaphosphate was made up. Varying quantities were added to calibrated tubes so that the final concentrations after blood was added ranged from 0.1 g per 100 cc to 2.0 g per 100 cc. Blood was allowed to flow directly from the sheep being used into the tubes. Each tube was rocked carefully to insure complete mixing. A control tube containing no reagent was treated in the same way. The control tube showed clotting in 5 minutes. All tubes containing sodium hexametaphosphate remained unclotted. Experiments were also performed on human blood with the same results. Concentrations of sodium hexametaphosphate less than 0.1 g per 100 cc slowed the process of clotting but did not prevent it entirely as was the case of the higher concentrations.

It is well known that the use of excess quantities of an anticoagulant such as sodium oxalate interferes with certain analytical procedures such as deproteinization. Folin-Wu filtrates were made on the samples but no interference with successful deproteinization was detected at any of the concentrations used.

These experiments indicate that sodium hexametaphosphate can be added to the list of blood anticoagulants, and may have certain advantages over existing agents.

11525 P

Barium in the Mammalian Retina.*

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Ramage and Sheldon¹ discovered the existence of Ba in the chorioid of ox eyes. They believed that Ba increased in quantity with age and report that it is not present in calves' eyes in sufficient quantity to be detected by their method of flame excitation of the spectrum. Furthermore they say that Ba is not present in the chorioids of human, sheep, pigs, horses, dogs and many sea fish. Ramage and Sheldon failed to find this element in the retina although they could detect it in the iris and the pigment of the chorioid. It is of some significance that they found Ba in the chorioids of all neat cattle beyond 3 years in age. Gerlach and Müller² examined eyes from a wide variety of animals including man and discovered Ba in the chorioid of most of them. It was not present uniformly in human chorioids and there appear to be no age peculiarities in its distribution. These writers also describe Ba in the retinae of ostriches, rabbits, cats, cattle and in one human.

The material in the present series consisted of 19 pigs, 17 ox, 24 sheep and 12 kitten eyes. The spectrographic method used was that described by us in an earlier paper (Scott and Canaga³). We used as identifying lines the 4535.5 and 4934.1 Å. These lines are quite sensitive and can be definitely located with little trouble. An

* Aided by a grant from the Josiah Macy, Jr., Foundation.

¹ Ramage and Sheldon, *Nature*, 1931, **128**, 376.

² Gerlach and Müller, *Arch. f. path. Anat. u. Physiol.*, 1936, **296**, 588.

³ Scott and Canaga, *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 275.

attempt was made to localize Ba in the tissue by separating the retina into 2 layers. Experiments showed that the retina split easily along the internal limiting membrane giving one layer which consisted largely of nerve fibers and another which was made up of pigmented epithelium, rods and cones and their nuclei, neuroglial and bipolar cells.

Barium was observed in all specimens examined and was apparently evenly distributed throughout the substance of the retina at least insofar as our rough localization permitted us to estimate. The presence of pigment in the one layer apparently had little or nothing to do with the amount of Ba found. Previously mentioned work seemed to suggest that Ba was associated with the pigment. Spectrographic studies indicate that Ba is not found in any appreciable quantities in other tissues, certainly not in the quantities observable in the retina. Our own experience with some hundreds of samples supports this statement. In fact only one instance is on record in our files and that is the consistent finding of Ba in skeletal muscle the nerve supply of which had been severed 6 months before the examinations were made. In these samples only traces of Ba could be found.

Since a large number of our series consisted of retinae from neat cattle it is of some interest that Blumberg and Rask⁴ found traces of Ba in milk. However, Ramage and Sheldon state that they have been advised that Ba is more apt to be found in milk which has soured in glass containers. We do not attach much significance to this in relation to Blumberg and Rask's findings as they used pyrex glassware throughout. Our experiments involved digesting the retinae and other tissues in nitric acid in pyrex vessels. Only the retinae showed Ba in their spectra. Evidently, therefore, an element, quite active photoelectrically, is present in tissue wherein light is translated into nerve impulses.

⁴ Blumberg and Rask, *Nutrition*, 1933, **6**, 285.

11526 P

Mean Molecular Weights of Synthetic Mixtures of Bovine Plasma Albumin and Globulin.

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Albumin and globulin, though generally considered to be separate entities, show sedimentation anomalies when they are studied in mixtures with the ultracentrifuge (Pederson¹). The data of McFarlane² suggest that globulin may dissociate in such mixtures. We have studied purified bovine plasma albumin and globulin, alone and in synthetic mixtures, by osmometric methods.

Sterile bovine plasma was carefully fractionated with ammonium sulfate at 0°C by a technic similar to that used by McMeekin.³ The albumin and globulin fractions were reprecipitated, freed of ammonia by dialysis and then dialyzed to equilibrium with phosphate buffer of pH 7.4, $\mu = 0.16$. Osmotic pressures were measured at 0°C by the method to be described by Keys. Protein concentrations were determined with the Pulfrich refractometer in every sample and checked by the micro-Kjeldahl method of Keys⁴ in about every third sample. Mean molecular weights were determined by the method of Adair and Robinson⁵ in which the pressure-concentration ratio is extrapolated at zero concentration. At least 4 acceptable P/C values at below 3% protein concentration were obtained for each sample.

The molecular weights of the albumin and globulin used here were 69,900 and 170,000, respectively, and these values were used to compute the theoretical mean mol. wts. of the mixtures from Dalton's law of partial pressures. The observed mol. wts. of the mixtures deviated widely and systematically from the simple predictions. As shown in Fig. 1, the mean mol. wts. of the mixtures are lower, and the osmotic pressures are higher than predicted. The deviation approaches a linear function of the mol. wt. in the range of A/G from 0.1 to 2.1. Similar results were obtained with other preparations.

¹ Pederson, K. O., *Compt. Rend. Lab. Carlsberg, Copenhagen*, 1938, **22**, 426.

² McFarlane, A. S., *Biochem. J.*, 1935, **29**, 407.

³ McMeekin, T. L., *J. Am. Chem. Soc.*, 1939, **61**, 2884.

⁴ Keys, A., *J. Biol. Chem.*, 1940, **132**, 181.

⁵ Adair, G. S., and Robinson, M. E., *Biochem. J.*, 1930, **24**, 1864.

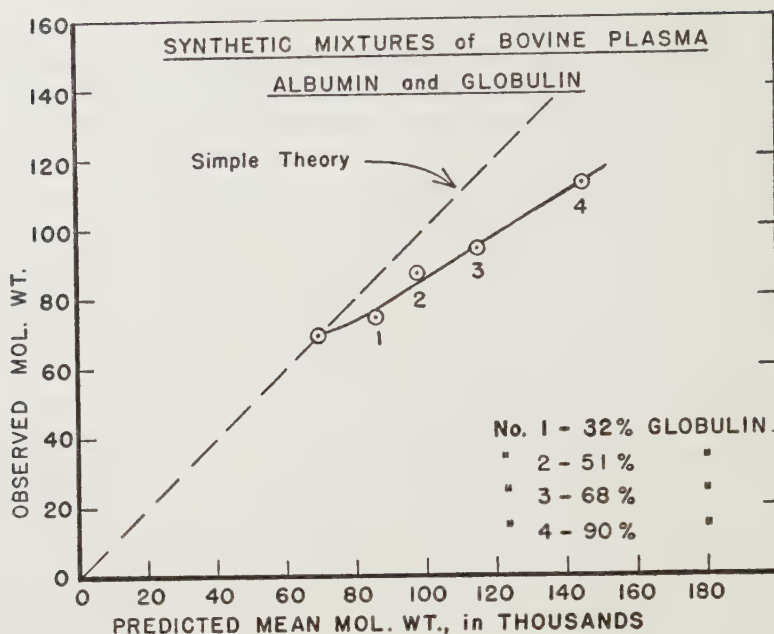


FIG. 1.

Sedimentation diagrams of such mixtures clearly show 2 fractions corresponding in molecular size with albumin and globulin but McFarlane's (op. cit.) data on horse plasma proteins indicate that the proportion of the smaller molecules is higher than the composition of his mixtures would warrant. This strongly reinforces our conclusion that globulin dissociates in the presence of albumin. Assuming that the dissociation product is a particle like albumin in size, our results would indicate that about 35% of the globulin undergoes dissociation; in other words, in an albumin-globulin mixture, for every globulin 170,000 molecule, there would be a globulin 70,000 molecule. Alternatively, globulin 170,000 may split into equal halves (mol. wt. 85,000, cf. McFarlane, op. cit.).

These results explain the divergent values reported for the mol. wt. of globulin—145,000 to 192,000. The presence of minute amounts of albumin depresses the mean mol. wt. to a remarkable degree. It is not easy to separate globulin cleanly from albumin; on the other hand, we have had no difficulty in preparing pure albumin. It appears that the formation of dissociated globulin is reversible, that the dissociated globulin salts out with the other globulin and it becomes associated when it enters an albumin-free phase. These phenomena are most striking at very low A/G ratios and are seen in purest form at infinite dilution, but they are de-

monstrable at an A/G ratio of 2.1 and protein concentrations of over 3%. In human plasma ($A/G = 2$, tot. prot. = 7%) the effect is sufficiently obscured so that the simple relation of Keys⁶ holds approximately.

11527 P

Skin Sensitization to a Simple Compound by Injections of Conjugates.

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In view of a recent paper¹ touching upon the subject, we wish to make a preliminary communication of a study under way for some time on the possibility of producing in animals skin sensitivity to drugs by immunizing with conjugates. We have in fact been able to render guinea pigs sensitive to superficial application of picryl chloride^{2, 3} by intraperitoneal injections of a conjugate resulting from the treatment of guinea pig erythrocyte stromata with picryl chloride in alkaline solution, killed tubercle bacilli as in previous work⁴ having been injected beforehand.

Since even minute quantities of the simple substance can sensitize under certain conditions and must be avoided, the chief concern in these experiments was to guard against the inclusion of unchanged picryl chloride in the injection material. This was carried out by adding an excess of glycine which removed any possible remainder of the substance through chemical combination, and by washing with aqueous alcohol.

The large majority of animals treated in this way have shown upon subsequent testing with the simple substance typical reactions of the contact dermatitis type.

⁶ Keys, A., *J. Phys. Chem.*, 1938, **42**, 11.

¹ Haxthausen, H., *Acta Derm.-Vener.*, 1940, **21**, 158.

² Landsteiner, K., and Jacobs, J., *J. Exp. Med.*, 1935, **61**, 643.

³ Landsteiner, K., and Chase, M. W., *J. Exp. Med.*, 1937, **66**, 337.

⁴ Landsteiner, K., and Chase, M. W., *J. Exp. Med.*, 1940, **71**, 237.

Osmotic Activity Changes of Serum and Salt Solutions Placed in the Gall Bladder.

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Dreser,¹ the first to use cryoscopy on animal fluids, made the first determination of the osmotic pressure of bile. Numerous early investigators, among them Brand,² Strauss,³ Bernstein,⁴ Bosquet,⁵ Koziezkowsky,⁶ Messadaglia and Colletti⁷ determined cryoscopically the osmotic pressure of animal and human bile obtained by various methods from living and dead specimens. They came to the conclusion that the osmotic pressure of bile, both bladder and hepatic, was approximately the same as the osmotic pressure of the blood of the same animal species; *i.e.* the depression of freezing point of the bile and blood both lay in the same range (about -0.54°C to -0.58°C for most species). Of the many objections to the earlier work, the varied methods of collection and the inaccuracy of the cryoscopic technic are perhaps the most significant. A difference in freezing point of 0.01°C corresponds to a difference in osmotic pressure of nearly 100 mm of Hg. Ravdin, *et al.*,⁸ state that despite the wide variance of constituents, the osmotic pressures of hepatic and bladder bile, as determined by the depression of freezing point, are approximately the same; and that the total depression of freezing point may be accounted for on the basis of the osmolar concentration of base, chloride, and bicarbonate present. Yet the difference in osmotic pressure of their hepatic and bladder bile amounts to 357.2 mm of Hg. They also conclude that the osmotic pressure of hepatic and bladder bile is approximately the same as that of serum. On the basis of experiments in which they placed various bile constituents individually into a bile-free dog's gall bladder, Ravdin *et al.*⁹ came to the conclusion that regardless of the concentration of the original solution, the total osmolar concentration of the fluid in the gall

¹ Dreser, *Arch. f. exp. Path. u. Pharm.*, 1892, **29**, 303.

² Brand, *Arch. f. d. gesammte Physiol.*, 1902, **90**, 491.

³ Strauss, *Berl. Klin. Wehnsch.*, 1903, **40**, 261.

⁴ Bernstein, *Arch. f. d. gesammte Physiol.*, 1905, **109**, 207.

⁵ Bosquet, cited by Strauss.

⁶ Koziezkowsky, cited by Strauss.

⁷ Messadaglia and Colletti, cited by Strauss.

⁸ Ravdin, Johnston, Riegel and Wright, *Am. J. Phys.*, 1932, **100**, 317.

⁹ Ravdin, Johnston, Austin and Riegel, *Am. J. Phys.*, 1932, **99**, 638.

bladder after a period of time approaches that of serum. Reinhold and Wilson¹⁰ state that: "Although the sum of the molar concentrations of anions and cations in bile exceeds that in serum, the osmotic pressure of the two fluids, as shown by the work of others, is practically the same. Actually the molar concentrations of inorganic ions are approximately the same in both. It would appear, therefore, that the principal organic ion of dog bile, taurocholic acid, either exhibits little osmotic activity or diminishes the osmotic activity of other ions." Gilman and Cowgill¹¹ using Hill's method found approximate isotonicity of blood and hepatic bile, and that artificially produced changes in the osmotic pressure of the blood produced parallel changes in the osmotic pressure of hepatic bile. Their osmotic pressure values are given in terms of milliequivalents of an osmotically equal NaCl solution. They state they are "confident the values for osmotic pressure—are accurate to within 1 milliequivalent;" yet their average values for the osmotic pressure of blood and hepatic bile under all conditions are 155 and 151 milliequivalents, respectively. Therefore the difference in the average osmotic pressure of blood and bile falls 3 milliequivalents outside the range of experimental error. Furthermore, the values for hepatic bile were 2-9 milliequivalents lower than for blood in 13 of 15

OSMOTIC ACTIVITY AND CHLORIDE CONCENTRATION CHANGES IN THE GALL BLADDER.

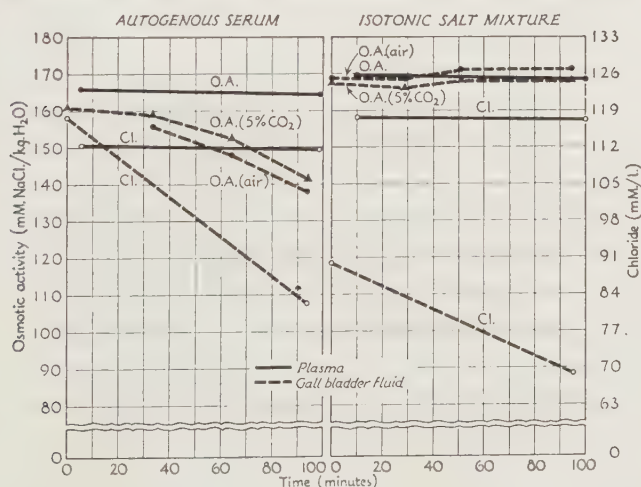


FIG. 1.

¹⁰ Reinhold and Wilson, *Am. J. Phys.*, 1934, **107**, 378.

¹¹ Gilman and Cowgill, *Am. J. Phys.*, 1933, **104**, 476.

cases. In the other 2 cases the value for bile was 1 milliequivalent higher than for blood. In order to investigate the role of the gall bladder in osmotic processes, it was decided to study the changes in osmotic activity of serum and nearly isotonic $\text{NaCl} + \text{Na}_2\text{SO}_4$ solutions placed in the gall bladder by the vapor tension method.

Methods. Cats anesthetized with nembutal were used as experimental animals. The gall bladder was entered via a whistle-tip fiber ureteral catheter so inserted and tied into the cystic duct as not to injure the cystic vessels. Injections into and withdrawals from the gall bladder were then accomplished by a syringe whose needle fit closely into the open end of the catheter. When not in use, the open end of the catheter was closed by a piece of wire of similar diameter to the bore of the catheter. After the bladder bile was removed, the gall bladder was washed out several times with normal saline at body temperature then with the fluid to be injected at body temperature. Finally such an amount of fluid was injected as to moderately distend the gall bladder (usually 1.5 to 0.7 cc). The fluids injected were: (1) the cat's own serum obtained just before injection from femoral vein blood, and (2) $\text{NaCl} + \text{Na}_2\text{SO}_4$ (in about equiosmotic proportions) solution approximately isotonic with the cat's plasma. Small samples of gall bladder fluid were withdrawn about every $\frac{1}{2}$ hour for $1\frac{1}{2}$ to 2 hours. Blood plasma samples were taken as described by Roepke and Visscher¹² at the beginning and end of the experiment. All samples were protected from evaporation and CO_2 loss. Osmotic activity (see Roepke and Visscher¹²) determinations were made with Hill's thermoelectric method as modified by Baldes.^{13,14} Vapor tension measurements were made with air and CO_2 mixtures in the thermocouple chamber in order to control the influence of the CO_2 tension. Chloride was determined according to the method of Van Slyke.¹⁵

Results. Fig. 1 shows the typical results obtained on placing 1 cc of autogenous serum in a cat's gall bladder. Within 90 minutes the volume decreases (50% to nearly 100%); the chloride falls (30% to 40%); and the osmotic activity decreases (7 to 23 mM). The osmotic activity is higher in 5% CO_2 than in air. The osmotic activity and chloride concentration of the blood remain relatively constant. If the serum is poisoned with .004 M HgCl_2 ; the volume remains practically constant, the chloride decreases (but only about

¹² Roepke and Visscher, *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 500.

¹³ Baldes, *J. Sc. Instruments*, 1934, **11**, 223.

¹⁴ Baldes and Johnson, *Biodynamics*, 1939, No. 47, 1.

¹⁵ Van Slyke, *J. Biol. Chem.*, 1923, **58**, 523.

15%), and the osmotic activity does not change. The osmotic activity and chloride concentration of blood again remain nearly constant.

Fig. also shows the results of an experiment in which 1 cc of an isotonic mixture of osmotically equal parts of $\text{NaCl} + \text{Na}_2\text{SO}_4$ was placed in a cat's gall bladder. The volume decreased (50% to 75%). The chloride (25% to 50%). The osmotic activity usually rose somewhat (3 to 5 mM), and was higher in air than in 5% CO_2 . The osmotic activity and chloride concentration of the blood remained relatively constant. If the salt solutions are markedly hyper- or hypotonic to the blood (8 to 10 mM), the osmotic activity of the gall bladder fluid decreases or increases, respectively, to approach that of blood. In these cases there is also a decrease in volume and chloride. If the salt solution is poisoned with .001 M HgCl_2 ; the volume increases (50%), the chloride increases (30%), and the osmotic activity increases more rapidly and markedly (8 mM). The osmotic activity of the plasma remains unchanged.

The osmotic activity of the removed gall bladder bile was usually 1 to 3 mM lower than that of blood removed 15 to 30 minutes later.

11529 P

Cultivation of the St. Louis Encephalitis Virus.*

ELEANORA MOLLOY. (Introduced by C. W. Jungeblut.)

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The virus of St. Louis encephalitis is known to grow readily on the chorioallantoic membrane of the developing hen's egg and in tissue cultures of the Li and Rivers type.¹⁻⁴ In both media, however, virus titrations have been uniformly low, usually attaining levels of 10^{-2} , with an occasional maximum of 10^{-3} .

Successful propagation of the lymphogranuloma venereum virus

* This research was supported by a grant from the W. J. Matheson Fund for the study of encephalitis.

1 Syverton, J. T., and Berry, G. P., *Science*, 1935, **82**, 596.

2 Harrison, R. W., and Moore, E., *PROC. SOC. EXP. BIOL. AND MED.*, 1936, **35**, 359.

3 Schultz, E. W., Williams, G. F., and Hetherington, A., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, **38**, 799.

4 Smith, M. G., and Lennette, E. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 323.

was obtained by Sanders⁵ with the use of a new type of medium consisting of tissue elements in ox serum ultrafiltrate.⁶ Higher yields of virus were obtained at room temperature than at 37°C. The stability of these cultures and their marked potency, together with the fact that serum ultrafiltrate is protein-free made it appear desirable to apply this method to the propagation of St. Louis encephalitis virus.

Tissue cultures were prepared in rubber stoppered 50 cc Erlenmeyer flasks by adding minced embryonic mouse brain to 10 cc of serum ultrafiltrate diluted 1 in 3 with Simms' salt solution.⁵ These flasks were inoculated with 0.1 cc of a 1:10 mouse brain virus suspension† and parallel series maintained, one at room temperature and one at 37°C. Passages were made by transferring 0.1 cc of the clear supernatant fluid every 5 days. After intervals of 5, 10 and 15 days' incubation, potency tests were carried out by intracerebral inoculation of groups of 4 mice (8-12 g) with 0.03 cc of serial tenfold dilutions of the supernatant fluid. The endpoint in these titrations was taken as the last dilution causing characteristic symptoms and death in 50% of the inoculated mice. The identity of the virus was assured by two neutralization tests with a known antiserum, carried out with the 5th and 28th culture passages.

During 28 culture generations, the virus titers have been consistently higher after incubation at room temperature (10^{-5}) than at 37°C (10^{-3}). These titers, once attained, were maintained at a constant level during the first 10 days, but showed a drop in potency to 10^{-1} , 10^{-2} after 15 days. No difference was observed between the virus content of the whole culture (emulsified tissue plus supernatant fluid) and that of the clear supernatant fluid alone.

When embryonic guinea pig brain was used in place of mouse brain the virus grew readily at room temperature (10^{-2} , 10^{-3}), but showed a tendency to die out after 4 to 7 passages in cultures of other organs (liver, lung, spleen, kidney, heart, intestine). After 12 passages through embryonic guinea pig brain cultures the virus still failed to infect guinea pigs.

Cultures of adult mouse organs (brain, liver, kidney, spleen, heart, adrenal) have uniformly failed to support the growth of this virus in repeated tests, irrespective of variations in technic, such as temperature, amount of tissue, amount of fluid, method of transfer.

An attempt was made to combine the tissue culture technic with

⁵ Sanders, M., *J. Exp. Med.*, 1940, **71**, 113.

⁶ Simms, H. S., and Stillman, N. C., *J. Gen. Physiol.*, 1937, **20**, 603.

† The strain used was isolated in 1933 by Dr. M. Holden of this Department.

advantages offered by the chorioallantoic membrane of the developing egg. The chorioallantoic membrane of a 10-day-old egg was removed, washed in saline, and placed in 10 cc of serum ultrafiltrate in a 50 cc Erlenmeyer flask. The cultures were incubated at room temperature and the supernatant fluid only was used for passage and titration. The virus titered up to 10^{-4} after six passages and up to 10^{-5} after nine and ten generations. With this type of culture, only very fresh preparations were used, as, on storage, acid accumulates which must be neutralized by alkali in order to maintain a constant pH over a long period of time. The standardization of this type of culture is under consideration at present.

Conclusions. The virus of St. Louis encephalitis may be grown in a medium containing embryonic mouse or guinea pig brain in ox serum ultrafiltrate. Cultures of organs from adult mice fail to support growth of the virus. Incubation at room temperature produces higher titers (10^{-5}) than incubation at 37°C (10^{-8}). At both temperatures the attained titer remains almost unchanged for 10 days but shows a decrease after 15 days' incubation. The virus is present in the same concentration in the supernatant fluid as in the emulsified whole culture. Infected chorioallantoic membranes maintained in serum ultrafiltrate at room temperature support growth of this virus up to titers varying from 10^{-4} to 10^{-5} .

11530

Search for Microorganisms of the Pleuropneumonia Group in Rheumatic and Non-Rheumatic Children.

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It has recently been demonstrated¹ that mice of various stocks are carriers of a new group of filtrable microorganisms which biologically can be classed with the causative agent of *pleuropneumonia boorum* but otherwise is quite distinct as regards pathogenicity, affinities for special cell types *in vivo*, and immunological identity. In mice, these microorganisms are usually found in association with the epithelium of the conjunctiva and nasal mucosa without giving rise to any signs of disease. However, when cultures of certain

¹ Sabin, A. B., *Science*, 1939, **90**, 18.

types of these microorganisms are injected intravenously or by certain other routes, experimental diseases are produced in mice which resemble in many respects some of the manifestations of rheumatic fever and rheumatoid arthritis in man.² Many attempts have been made, therefore, to isolate similar microorganisms from these human diseases. Failure to obtain such microorganisms from the exudates and tissues of a small number of patients with rheumatoid arthritis or rheumatic fever has already been reported¹⁻³ and the purpose of the present investigation was (a) to determine whether or not human beings may be carriers of similar or related microorganisms, and (b) to study additional material from patients with rheumatic fever or rheumatoid arthritis.

Cultures were obtained from the nose and throat and in most instances also from the conjunctiva of 100 human beings, 95 of whom were under 15 years of age. Material obtained with sterile cotton swabs was streaked on agar plates containing 30% ascitic fluid. After 4 days' and again after 7 days' incubation at 37°C, all plates were examined with the microscope at a magnification of 100 times and a thorough search was made for colonies which might resemble even remotely those of the pleuropneumonia group. No such colonies, however, were found in any of the cultures. Among the patients that were examined in this manner there were 28 in the active phase of rheumatic fever, 2 in the active stage of Still's disease, 14 with various types of infection of the upper respiratory tract (mostly pharyngitis or tonsillitis associated with otitis media), 3 with pneumonia, and most of the remainder with miscellaneous medical or surgical conditions.

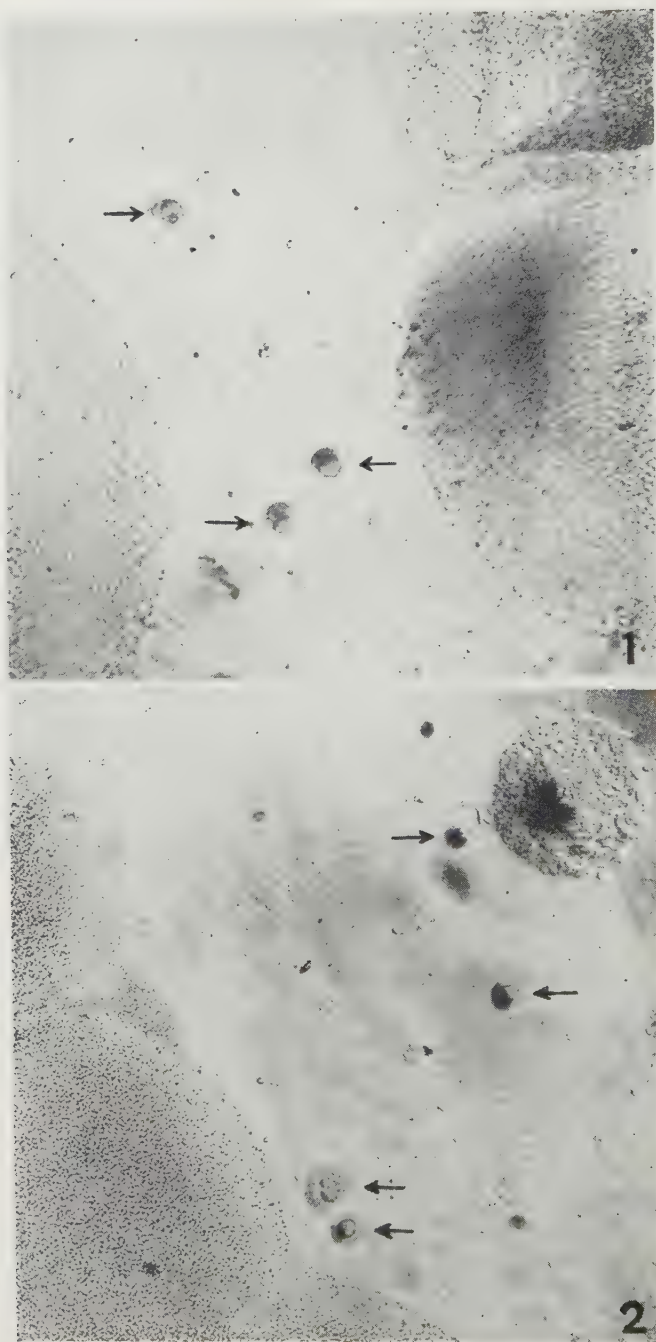
The blood of 9 children with rheumatic fever (acute febrile phase) and of two children during the febrile stage of Still's disease was cultured by adding 5 cc to 25 cc of broth containing 30% ascitic fluid and 0.5% glucose. No growth was obtained despite prolonged incubation and "blind passage." Exudates from the knee-joints of 3 children during their first attack of rheumatic polyarthritis were similarly cultured on fluid and solid media, but without obtaining any growth. Pericardial fluid, the pericardium and myocardium, and vegetations from the mitral valve obtained at necropsy from two children who died with active rheumatic carditis also yielded no growth.

Because experience with the pleuropneumonia group in mice indicated that they may often be intimately associated with the affected

² Sabin, A. B., *Science*, 1938, **88**, 575; *ibid.*, 1939, **89**, 228.

³ Findlay, G. M., Mackenzie, R. D., and MacCallum, F. O., *Brit. J. Exp. Path.*, 1940, **21**, 13.

cells, and since the carrier state in mice was established by streaking the conjunctiva and nasal mucosa rather than exudates from those sites, it was decided to investigate a series of tonsils removed from children for various reasons. A piece of tissue was removed from each tonsil, minced to expose a larger surface, and streaked on a 30% ascitic fluid agar plate. The tonsils of 58 children (116 specimens) were thus examined, and in 3 cases there were colonies, 20 to 40 μ in size, bearing a striking resemblance to those of certain members of the pleuropneumonia group. The appearance of these colonies (to be referred to as "X" colonies) is illustrated in Figs. 1 and 2. They always occurred along the streak either independently of the adjacent bacterial colonies (Figs. 1, 2) or at the border of and in intimate association with a bacterial colony. The plates were examined routinely 4 and 7 days after incubation; in 2 cases the "X" colonies were seen on the 4th day and in the third not until the 7th day, although in a repeat culture from the same tonsil many colonies appeared on the 4th day. Impression films of zones containing these colonies were unsatisfactory because the bacteria from the adjacent colonies obscured the field. Many attempts to passage the "X" colonies in series were without success. When an isolated "X" colony was streaked on 30% ascitic fluid agar or put into broth containing 30% ascitic fluid and 0.5% glucose no growth of any kind occurred. When "X" colonies and adjacent bacterial colonies were passaged together only the bacterial colonies grew out. In 2 of the 3 cases it was possible to obtain "X" colonies several times by repeating cultures from the same tonsils which were kept in the refrigerator, but passage was invariably unsuccessful. There was thus no evidence that the "X" colonies were either a variant or a symbiont of any of the tonsillar bacteria, and their nature remains obscure. It is perhaps significant that they were not observed once among the "swab" cultures from the eyes, nose, and throat of the 100 cases studied by the same method. Swabs from the nose and tonsillar regions of the child, whose tonsils yielded the largest number of "X" colonies, were cultured 7 weeks after tonsillectomy but no "X" colonies were found. The possibility must be investigated that the "X" colonies may represent pleuropneumonia-like microorganisms which are intimately associated with certain cells and have such specific growth requirements that only one generation is possible on the 30% ascitic fluid agar, but it should be stressed that there is still no evidence that there is a human group of pleuropneumonia organisms such as has been shown to exist in cattle, sheep and goats, dogs, rats, and mice.



FIGS. 1 AND 2.

Growth resulting from streaking human tonsils on 30% ascitic fluid agar. Arrows point to "X" colonies. Fig. 1—X112; Fig. 2—X150.

Summary. Cultures on 30% ascitic fluid agar of material obtained by swabbing the eyes, nose, and throat of rheumatic and non-rheumatic children and a few adults failed to reveal any pleuropneumonia-like colonies. No success was encountered in additional attempts to isolate microorganisms of the pleuro-pneumonia group from the blood of children in the febrile phase of acute rheumatic fever or Still's disease, from the joint fluid during the first attack of rheumatic polyarthritis, and from rheumatic pericardial, myocardial, and valvular tissues obtained at necropsy. Cultures of 58 pairs of excised tonsils, however, yielded in 3 cases peculiar microscopic colonies ("X" colonies) which were 20 to 40 μ in size and strikingly similar to those of certain members of the pleuropneumonia group. The "X" colonies could not be passaged beyond the first generation, and their nature remains unknown.

11531 P

Pathogenic Pleuropneumonia-Like Microorganisms in Tissues of Normal Mice and Isolation of New Immunological Types.

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That normal mice can be carriers of a distinct group of pathogenic pleuropneumonia-like microorganisms has already been demonstrated in an investigation of 3 different stocks of animals in New York.¹ Previous studies have established that their natural habitat was the conjunctiva and nasal mucosa,¹ although at least one strain was found in the brain of a normal mouse.² They have also been isolated from the lungs of mice which had received nasal instillation of various materials under ether anaesthesia^{1, 3} and in the brains of mice which had been used for passage of various other infectious agents.^{2, 4} Three distinct immunological types—A, B, and C—which vary in their pathogenicity and tissue affinities as well as in their antigenic make up, have now been described.

¹ Sabin, A. B., *Science*, 1939, **90**, 18.

² Sabin, A. B., *Science*, 1938, **88**, 575; *ibid.*, 1939, **89**, 228.

³ Sullivan, E. R., and Dienes, L., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 620.

⁴ Findlay, G. M., Klieneberger, E., MacCallum, F. O., and Mackenzie, R. D., *Lancet*, 1938 (Dec. 31st), 1511.

The purpose of the present study was to determine (a) to what extent these microorganisms were present in other tissues of carrier mice, (b) whether the carrier state persisted throughout life or was limited to a special age group, and (c) to investigate further the multiplicity of immunological and biological types that make up the mouse pleuropneumonia group. The mice used in the present studies came from an albino stock that had been inbred in Ohio for about 50 years. A preliminary investigation of the nasal mucosa and conjunctiva of 6 mice, yielded 5 new strains from 3 mice, all of which produced the neurotropic exotoxin and were immunologically type A. The distribution of the microorganisms in various tissues of carrier mice was studied in 10 animals which were 3 to 4 weeks old. The eyes, nose, trachea, lungs, heart, blood, liver, spleen, kidney, brain, and intestinal contents were cultured on 30% ascitic fluid agar. The intestinal contents were taken up in physiological salt solution, centrifuged at about 2000 rpm for 30 minutes, and the supernatant liquid was used for cultivation. Microorganisms of the pleuropneumonia group were obtained from 7 of the 10 mice (Table I). With the exception of the eyes and the upper respiratory tract they were isolated from the brain of 3 of these mice. Only a

TABLE I.
Pathogenic Pleuropneumonia-like Microorganisms in Various Tissues of Carrier Mice.

Mouse No.	Tissues cultured									
	Eyes	Nose	Trachea	Lungs	Heart blood	Liver	Spleen	Kidney	Intestinal contents	Brain
1	0	+	0	0	0	0	0	0	0	0
		(A)*								
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	+
										(A)
4	0	+	0	0	0	0	0	0	0	0
5	+	+	+	0	Uns.	Uns.	Uns.	Uns.	0	0
		(A,D,E)								
6	+	+	0	0	0	0	0	0	0	+
	(A)									(A)
7	+	0	0	0	0	0	0	0	0	0
8	+	0	0	0	0	0	0	0	0	+
	(A)									(A)
9	0	0	0	0	0	0	0	0	0	0
10	0	Uns.	0	0	0	0	0	0	Uns.	0
11	0	+		+		0	0			0
		(D)		(D)						
12				0		0	0			+
										(D)

*Letters in parentheses refer to the immunological type of the strain that was isolated.

Uns.—culture unsatisfactory.

few colonies were present in the cultures from the brain (there were no ordinary bacteria) and all 3 strains proved to be neurotropic exotoxin producing Type A's. Because the culture obtained from the nose of mouse 5 behaved peculiarly in tests for pathogenicity and agglutination, it was plated out and 3 different kinds of pleuropneumonia-like colonies were observed. Isolation and passage of single colonies revealed that the original culture was a mixture of 3 immunologically distinct types—A, D, and E. The new types D and E produce a progressive chronic arthritis but no neurotropic exotoxin and are immunologically different not only from one another but also from types A, B, and C of the mouse group and L₃ and L₄ of the rat group of pleuropneumonia-like microorganisms. Mice 11 and 12 (Table I) were sacrificed several days after the intravenous injection of bacteria, and the 3 strains of pleuropneumonia-like microorganisms which were isolated from the nose, lungs, and brain all belonged to the new type D.

Cultures from the conjunctiva and nasal mucosa of 10 old mice (6 months or older) yielded 6 strains from 4 mice, suggesting that the carrier state is probably not a transitory phenomenon. Two of these strains were typed and the one from the eye was a type A and that from the nose a type B. While type A as well as other types have been encountered in various tissues, the strains which have thus far been isolated from the eyes have all been type A. That the carrier state probably develops after birth by contact infection is suggested by a preliminary study of 5 mothers and their offspring. While 4 of the 5 mothers were carriers (nose, eyes, or both), no such microorganisms were found in 13 of their offspring at 3 days of age and were present in the nose of only 1 out of 20 at 5 days of age.

Summary. Pathogenic microorganisms of the mouse pleuropneumonia group in addition to being present in the conjunctiva and nasal mucosa may often be found in the brain and occasionally also in the trachea and lung of normal carrier mice. They were not found in the heart blood, liver, spleen, kidneys or in the intestinal contents. The carrier state is probably the result of contact infection and has been demonstrated as early as the 5th day of life and later than 6 months. Two new, immunologically distinct types (D and E) have been isolated; they produce arthritis but not the neurotropic exotoxin which thus far has been found to be elaborated only by the type A strains.

Spread of Virus in an Unvaccinated Case of Human Rabies.

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A necropsy on an unvaccinated case of human rabies presented an opportunity to investigate several questions regarding the spread of the virus in human beings and the development of immune bodies during the course of the disease. Since rabies is noted for the centrifugal spread of the virus it was desirable to determine (particularly for correlation with poliomyelitis in man) to what extent the virus spread outwards along the olfactory pathway and whether or not it appeared in the feces in a case in which the portal of entry was known to be the hand.

The patient was a 55-year-old man who was bitten on the right hand by a dog. There was no treatment other than superficial cleansing of the wound. He died 2 months later after an illness of 2 to 3 days with a clinical diagnosis of rabies. Necropsy was limited to the head, and the following structures were investigated: (1) The skin and subcutaneous tissue at the site of the bite (which was still marked by hyperemic scar tissue) after superficial sterilization with iodine and alcohol, (2) upper cervical cord and medulla, (3) cornu ammonis, (4) olfactory bulbs, (5) nasal mucosa (mostly olfactory) removed after an intracranial exposure of the roof of the nasal cavity, (6) tonsillar and pharyngeal tissues, (7) saliva obtained by swabbing out the mouth and throat with sterile absorbent cotton, (8) feces removed from the rectum by spatula, (9) subarachnoid fluid over the exposed cerebral cortex removed with a needle and syringe, and (10) heart blood. The feces was prepared for inoculation in two different ways: (a) one 2 g sample was shaken with glass beads in 10 cc of broth and after horizontal centrifugation at about 2000 rpm for 15 minutes the supernatant liquid was passed through a Berkefeld "V" filter which had previously been saturated with 20 cc of broth; (b) a 2.5 g sample was shaken in 15 cc of distilled water and after centrifugation as above, the supernatant liquid was mixed with 2 cc of anaesthetic ether and thoroughly shaken for 10 minutes; after 5 hours in the refrigerator at approximately 5°C the mixture was again centrifuged and the very opalescent fluid portion removed. Preparation (a) was bacteria-free while preparation (b) was not. The saliva and mucus

were freed from the absorbent cotton with the aid of 30 cc of physiological salt solution. After horizontal centrifugation one part was injected into mice without any further treatment, another part was treated with 15% of anaesthetic ether in the same manner as the feces, and still another part was mixed with one-tenth its volume of rabbit serum and passed through a Berkefeld "V" filter which had been saturated with 10 cc of 10% rabbit serum in physiological salt solution. The other tissues were prepared as indicated in Table I. With the exception of the heart blood, subarachnoid fluid, and the centrifuged, untreated suspensions of the nasal mucosa and of the tonsillar and pharyngeal tissues, each specimen was injected into 6, two- to three-week-old white mice and one rabbit; where this number of animals does not appear in the table it means that they died within one or 2 days as a result of the inoculation.

The results shown in Table I indicate that while there was no demonstrable virus at the site of inoculation, *i.e.* the bitten area, there was enough present in the cervical cord and medulla, the cornu ammonis, and the olfactory bulbs to produce rabies in all the inoculated animals after a relatively short incubation period. The diagnosis of rabies was made by the demonstration of large numbers of typical Negri bodies in films of the brains of succumbing animals, by positive passage, and finally by neutralization with rabies immune serum which was kindly supplied by Dr. L. T. Webster of the Rockefeller Institute. A very small amount of virus, only enough to produce rabies in one of 6 mice, was present in the untreated, centrifuged suspensions of the nasal mucosa and of the tonsillar and pharyngeal tissues, while none was found in the Berkefeld "V" filtrates of the same preparations. Virus was also not demonstrated in the heart blood, subarachnoid fluid, saliva, and feces. The unaffected mice and rabbits were observed for 8 weeks and were then tested for immunity by an intracerebral injection of a 10^{-8} dilution of the virus (derived from the cord and medulla of this case and passaged in mice) which represented approximately 10 minimal mouse cerebral lethal doses. All of 62 test mice and 15 control mice of the same age developed rabies; 5 test rabbits and one control also succumbed.

With regard to the centrifugal spread of the virus along the olfactory pathways in human rabies, it would appear therefore that after entry by way of the nerves supplying the hand the virus can spread to and be present in appreciable amounts in the olfactory bulbs; the small amount which was demonstrated in the nasal mucosa indicates how little the extracranial part of the olfactory system can

TABLE I.
Distribution of Virus in an Unvaccinated Case of Human Rabies.

Material tested	Preparation for inoculation	Amt cc	Site	Animals inoculated	Result	Negri bodies	Passage
Skin lesion (site of bite)	Centrifuged suspension	.03 .5 .6	i. cer. " masseter	6 mice 1 rabbit	0,0,0,0,0,0 0		
Heart blood	-----	.03	i. cer.	6 mice	0,0,0,0,0,0		
Subarachnoid fluid over cortex	-----	.03	"	" "	0,0,0,0,0,0		
Upper cervical cord and medulla	10% susp.	.03 1.0 .5 2.5	" i. a. i. cer. i. a.	" " " " 1 rabbit	8*,10,10,11,11,11 CNS 8, D9+	+	+
Cornu ammonis	" "	.03 1.0	i. cer. i. a.	4 mice	13,13,14,14	+	+
Olfactory bulbs	Ground in 1 cc saline	.03 .5	i. cer. "	6 " 1 rabbit	12,12,12,13,13,14 CNS 10, D11	+	+
Olfactory mucosa	Centrif. susp.	.03	"	6 mice	14,0,0,0,0,0	+	+
10% suspension in 10% rabb. ser.	Berkefeld "V" filtrate	.03 1.0 .5 .5	" i. a. i. cer. masseter	6 " 1 rabbit	0,0,0,0,0,0 0	+	+

Tonsillar and pharyngeal tissues 10% suspension in 10% rabb. ser.	Centrif. susp. Berkefeld "V", filt.	.03 .03 1.0 .5 .5	i.cer. " i.a. i.cer. maseter	6 mice 6 " 1 rabbit	21,0,0,0,0,0 0,0,0,0,0,0 0	+	+
	Centrif. susp. Berkefeld "V", filt.	.03 .03 1.0 .5 4.0 1.0	i.cer. " i.a. i.cer. i.a. i.cer. i.a.	6 mice 6 " 1 rabbit 6 mice	(3 bact.); 1d?,0,0 0,0,0,0,0,0 0 0,0,0,0,0,0		
	Ether-treated	.03 1.0					
	Berkefeld "V", filt. Ether-treated	.03 1.0 .03	i.cer. i.a. i.cer.	6 " 6 " 6 "	0,0,0,0,0,0 0,0,0,0,0,0		
Feces	Berkefeld "V", filt.	.03	i.cer.	6 "	0,0,0,0,0,0		
	Ether-treated	.03	i.cer.	6 "	0,0,0,0,0,0		

*Numerals refer to day on which signs of nervous system involvement were first observed in mice. One of the earliest signs (which we have never observed in mice injected with other viruses) consisted of a momentary flaring of the ears when the mice were picked up with a forceps and dropped back into the cage.

+CNS 8, D9—Signs of nervous system involvement 8th day and dead 9th day.

Other abbreviations: i.cer. = intracerebral; i.a. = intraabdominal; 3 bact. = 3 mice died of bacterial infection; 1d? = mouse died on 19th day but because it had been chewed by its mates neither passage nor examination for Negri bodies or bacterial infection was possible.

be affected. While the failure to demonstrate virus in the saliva and feces may partly be due to inadequate methods, it may also be that there was insufficient centrifugal spread of the virus. The present status of the presence of virus in the salivary glands or saliva in human rabies is rather indefinite. Thus, Williams,¹ stated that "glands from human beings are seldom infective for test animals," and Leach² recently reported isolation of the virus from the salivary glands of only one of 3 human cases. Kraus, Gerlach, and Schweinburg³ pointed out the discordance in the results and opinions concerning infectivity of saliva, and added that no case of rabies has been known to be produced by the bite of a human being. Palawandow and Serebrennaja⁴ reported that they produced rabies in guinea pigs [the evidence is not unequivocal, however] by intramuscular injection of saliva from a 12-year-old girl with rabies, and according to Pawan⁵ the above authors obtained similar results with saliva from 5 rabid persons. Pawan⁵ tested the saliva of 6 persons with signs of paralytic rabies (Trinidad) by rubbing swabs moistened with saliva into the scarified abdominal wall of 7 rabbits, all of which became paralyzed and exhibited Negri bodies; by a similar method he demonstrated the virus in the saliva of bovines, horses, and vampire bats.

The other question to be investigated was whether or not immune

TABLE II.
Test for Neutralizing Antibodies Against Rabies Virus in Patient's Post-mortem Serum.

Dilution of virus	Result with		
	Brcth control	Patient's serum	Mouse immune sérum
10-2	5,5,5,6*	4,5,5,6	8,10,10,0
10-3	6,6,7,8	5,7,7,8	12,0,0,0
10-4	7,8,10,0	6,7,7,15	10,0,0,0
10-5	0,0,0,0	7,7,0,0	0,0,0,0
10-6	0,0,0,0	n.t.*	n.t.

*0.03 cc of the various dilutions of each mixture was injected intracerebrally into each of 4 mice. The numerals refer to the day on which signs of involvement of the nervous system (rabies) were first observed. n.t. = not tested.

¹ Williams, A. W., *Abt's Pediatrics*, 1925, **6**, 251.

² Leach, C. N., personal communication; see also Leach, C. N., and Johnson, H. N., Abstracts of communications, Third International Congress for Microbiology, 1939, p. 108.

³ Kraus, R., Gerlach, F., and Schweinburg, *Lyssa bei Mensch und Tier*, Vienna, 1926, pp. 80, 128-131.

⁴ Palawandow, H., and Serebrennaja, A. I., *Z. f. Immunitätsforsch.*, 1930, **68**, 236.

⁵ Pawan, J. L., *Ann. Trop. Med.*, 1937, **31**, 267.

bodies would be present in an individual who died 2 months after the introduction of rabies virus into the body. The question was of some interest first, because it is known that neutralizing antibodies appear in animals and human beings within 2 to 3 weeks after the injection of effective rabies vaccines, and second, because in diseases like equine encephalomyelitis and yellow fever in which the viruses are viscerotropic as well as neurotropic, neutralizing antibodies are often found in animals and human beings succumbing to the infection. The patient's serum obtained post-mortem was tested against the virus which was isolated from his spinal cord and medulla and passaged in mice 8 times; an immune serum from mice inoculated with a fixed strain of rabies virus (supplied by Dr. Webster) was tested simultaneously. Mixtures of equal parts of the test sera and various dilutions of the virus were injected intracerebrally in mice. The results, shown in Table II, indicate that the patient's serum had no neutralizing antibodies against the virus, while the mouse immune serum exhibited definite protection.

The results obtained in the present investigation throw some additional light on the behavior of rabies virus in man, but it will, of course, be necessary to carry out similar studies in other unvaccinated cases to establish whether or not the present findings are the exception or the rule.

Summary. In a 55-year-old man who died of rabies two months after a bite on the hand, virus was not demonstrated at the site of the bite but was present in appreciable amounts in the cervical cord and medulla, the cornu ammonis, and the olfactory bulbs; only a trace was found in the nasal mucosa and the tonsillar and pharyngeal tissue, and none was found in the feces, saliva, subarachnoid fluid, and heart blood. The patient's serum obtained post-mortem had no neutralizing antibodies for the virus.

11533 P

Plasma Protein Shifts During Diuresis.

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In a previous communication¹ changes were described in the plasma volume after the injection of salyrgan, aminophyllin, and digoxin. This report deals with the plasma protein changes under these conditions. Total proteins were determined by the Kjeldahl technic; albumin by the method of Campbell and Hanna;² plasma volumes by the method of Gregerson, Gibson and Stead³ as modified by Gibson and Evelyn.⁴

After the injection of salyrgan, as the plasma volume drops there is a concentration of proteins, with a rise in the percentage of total protein and a relatively greater increase in albumin, leading to an increase in the albumin:globulin ratio. When the plasma volume is markedly lowered following diuresis the percentage of plasma protein increases persist, but the total amount of circulating protein is conspicuously lowered.

Paralleling the increase in plasma volume after aminophyllin there is a fall in percentage of plasma protein, but an absolute increase in the amount of total circulating protein. The latter is largely accounted for by an increase in circulating albumin, resulting in an increase in the albumin:globulin ratio. During the subsequent drop, usually marked, in blood volume¹ there is a definite rise in percentage of total protein and albumin, though the amount of each in the total circulating plasma shows a striking drop.

When digoxin was used, the shifts in plasma protein were less regular, but in general as the plasma volume dropped there was a rise in the percentage of total protein and albumin, with little early change in the total amounts of each in the circulating plasma. When however the decrease in the plasma volume became marked, though the percentage values were maintained there was a large loss of protein from the circulating plasma.

¹ Calvin, D. B., Decherd, George, and Herrmann, George, *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 529.

² Campbell, W. R., and Hanna, M. I., *J. Biol. Chem.*, 1937, **119**, 15.

³ Gregerson, M. I., Gibson, J. G., and Stead, E. A., *Am. J. Physiol.*, 1935, **113**, 54.

⁴ Gibson, J. G., and Evelyn, K., *J. Clin. Invest.*, 1938, **17**, 153.

TABLE I.

Drug	Hr after injection	Plasma volume, cc	Total protein		Albumin		A/G ratio
			g%	g total circulating	g%	g total circulating	
Salyrgan MV	0	5140	5.74	295	3.13	162	1.20
	½	5110	5.91	302	3.26	166	1.23
	1	4620	6.02	279	3.45	159	1.34
	2	4620	6.11	283	3.46	161	1.31
	5	3280	6.20	205	3.63	119	1.42
Aminophyllin AO	0	6120	6.10	373	3.38	207	1.24
	½	6488	6.05	393	3.34	223	1.22
	1	6383	5.94	380	3.32	212	1.27
	2	6500	hemolysis				
	3	6697	6.01	402	3.39	227	1.30
	5	7202	5.87	423	3.43	242	1.41
	7	6278	6.25	393	3.63	228	1.39
	9	4913	6.39	314	3.62	178	1.40
	11	4072	6.44	263	3.81	156	1.45
Digoxin HB	0	3890	5.76	224	3.24	126	1.29
	1	4130	5.62	232	3.24	134	1.36
	2	3640	6.00	218	3.50	127	1.40
	3	3682	5.87	216	3.36	124	1.34
	5	3800	5.63	214	3.22	122	1.34
	7	3790	hemolysis				
	9	3060	5.94	182	3.63	111	1.57
	11	2505	6.06	152	3.65	92	1.51

Whenever there is an increase in the plasma volume after the injection of aminophyllin, there is noted an increase in the total circulating protein, chiefly albumin, similar to the changes observed⁵ in experimental hydremia in the dog. On the other hand, with each type of diuretic a drop in plasma volume is associated with moderate increase in the percentage of total plasma protein due largely to an increase in plasma albumin. The marked drop in the total circulating proteins under these circumstances seems explicable only on the assumption of plasma protein storage in the tissues.

⁵ Calvin, D. B., *Proc. Am. Physiol. Soc.*, in press.

Administration of Ascorbic Acid to an Alkaptonuric Patient.*

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Recently it has been reported from the senior author's laboratory that artificial alkaptonuria may be readily produced in the guinea pig by the supplementation of a vitamin C deficient diet with extra tyrosine.¹ With the subsequent administration of the vitamin the homogentisic acid in the urine was promptly reduced in amount and within 48 to 72 hr was completely absent. These findings suggested that the relation of ascorbic acid to the excretion of homogentisic acid by the alkaptonuric patient should be investigated. However, in the course of the experiments with the guinea pigs, the results of two such investigations appeared. These reports by Monsonyi² and by Diaz, Mendoza and Rodriguez³ indicate that ascorbic acid is without effect, but since, in our own more recent studies⁴ it has been apparent that in the guinea pig the effectiveness of the dose of ascorbic acid is dependent upon the state of vitamin saturation in the tissues, it was considered imperative to investigate the effect of doses of the vitamin greatly in excess of the normal human requirement, and also of the relatively small amounts used by the above investigators.

Experimental. The individual who served as a subject for this study is an essentially normal 65-year-old white male who exhibited at the time of these experiments alkaptonuria and ochronosis—a deposition of pigment mainly in cartilage which is characteristic of these individuals in later years. Since the patient had previously been on an experiment in which he consumed a relatively high intake of protein, he was continued on this level, the diet being a mixed diet with considerable variety from day to day but so planned that it

* Aided by a grant from the Committee on Scientific Research of the American Medical Association.

¹ Sealock, R. R., and Silberstein, H. E., *Science*, 1939, **90**, 517.

² Monsonyi, L., *Presse Med.*, 1939, **47**, 708.

³ Diaz, C. J., Mendoza, H. C., and Rodriguez, J. S., *Klin. Wchnschr.*, 1939, **18**, 965.

⁴ Sealock, R. R., Perkinson, J. D., and Silberstein, H. E., in press.

furnished approximately 130 g of protein per day. It included throughout the experiment 40 to 50 mg of vitamin C daily, an amount comparable to the previous daily intake of the patient. The urine was collected in 24 hr samples in bottles containing sufficient hydrochloric acid to make the sample slightly acid to congo red. The homogentisic acid was determined by the iodine-sodium thio-sulfate titration procedure of Metz⁵ as modified by Lieb and Lanyar.⁶ The ascorbic acid determinations were made in the usual fashion by titration of the fresh sample with standardized 2,6-dichloro-benzenone-indophenol. Since homogentisic acid also reduces the dye under the conditions of the titrations the values were corrected for the amount of the hydroquinone derivative.

Results. Following a control period vitamin C† was given orally at the comparatively high level of 1.0 g per day. As shown in Fig. 1, this amount for 4 days proved to be without effect on the excretion of the metabolite, as was the further administration on the 12th and

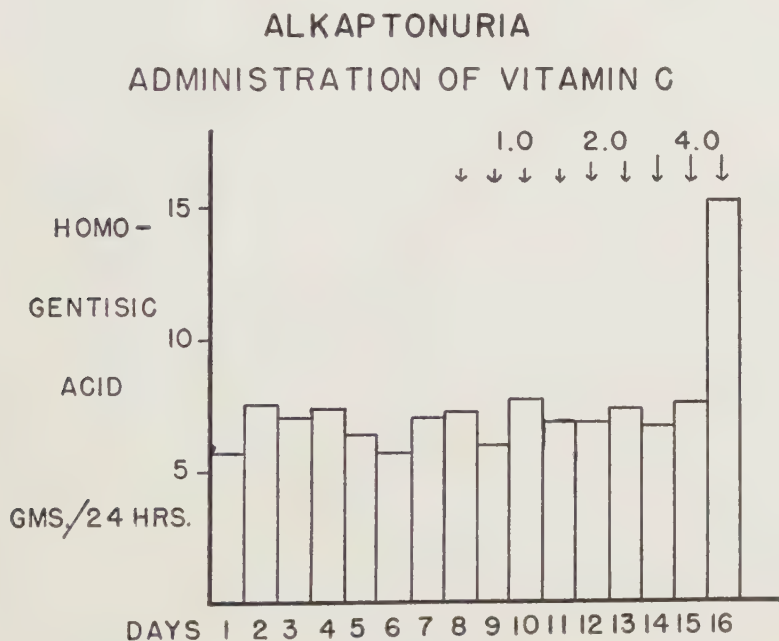


FIG. 1.

Mr. G—130 g protein per day. Beginning of last day also received 10 g of *l*-tyrosine. Ascorbic acid, g. —

⁵ Metz, E., *Biochem. Z.*, 1927, **190**, 261.

⁶ Lieb, H., and Lanyar, F., *Z. physiol. Chem.*, 1929, **181**, 189.

† The vitamin used in this study was very kindly supplied by Merck and Company, Incorporated, and Chas. Pfizer and Company, Incorporated.

13th days of 2.0 g in 0.5 g portions. For the following 3 days the ascorbic acid was again doubled, 2 of the 4 g being given by intravenous injections of 1 g each. That a high level of tissue saturation had been attained was indicated by the urinary excretion of 1.08, 1.62, 1.82 and 2.67 g of ascorbic acid on the 12th to the 15th days and a blood ascorbic acid of 2.89 mg per 100 cc. In view of the failure of even 4.0 g of the vitamin to effect the homogentisic acid output, the possibility yet remained of establishing a relationship between the two by flooding the metabolic processes with an extra dose of the precursor. In order to test this possibility 10 g of *L*-tyrosine were given at the beginning of the 16th day and subsequently four 1 g doses of ascorbic acid. The experiment resulted negatively, for in the 24 hr urine sample there were present 15.2 g of homogentisic acid, which when compared to the previous average represents a recovery of 88% of the theoretical.

The administration of the vitamin was not entirely without advantage, albeit the advantage was unrelated to the metabolism of the alkapton substance. Whereas the urine samples of the control period showed the usual tendency to darken on standing, with excretion of extra ascorbic acid there was no longer any discoloration even after several days, a finding which again illustrates the well known protective action of this substance against the oxidation of ortho- and para-diphenolic compounds by atmospheric oxygen.

It should be pointed out that the large doses of ascorbic acid were without visible effect on the ochronosis exhibited by the individual. However, these results do not preclude the possibility of a continued high intake of the vitamin in early life preventing the deposition of melanotic pigment in later years. That such a possibility is of some importance is evident from the fact that perhaps the only unpleasant feature of the condition is the appearance of melanin pigment in the cartilage of the ears and nose as discussed by Garrod.⁷

The ineffectiveness of ascorbic acid on the abnormal metabolism of the alkaptonuric individual indicates a real difference between this type of experiment and the one with the guinea pigs^{1,4} or in other words between hereditary and experimental alkaptonuria. When one recalls that the majority of metabolic reactions are chain reactions proceeding under the influence of many different factors and enzyme systems, the above difference is not surprising. In the guinea pig the missing factor is ascorbic acid while in the alkap-

⁷ Garrod, A. E., *Inborn Errors of Metabolism*, Oxford University Press, 1923, p. 58.

tonuric patient it is not the vitamin but some other factor as yet unknown.

Summary. The administration of from 1 to 4 g of ascorbic acid per day has been found to be without effect on the excretion of homogentisic acid by an alkaptonuric individual excreting an average of 7 g of the metabolite daily. The ineffectiveness of this excessive amount of vitamin is a finding which is in agreement with results with smaller doses obtained by other investigators.

The authors are indebted to Professor Vincent du Vigneaud for placing at their disposal laboratory facilities in the Department of Biochemistry of the Cornell University Medical College for the chemical work in connection with this experiment, and to Dr. Elaine P. Ralli of the Department of Medicine of New York University for the determination of the blood ascorbic acid.

11535 P

Relation of Pantothenic Acid to Dermatitis of the Rat.*

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Richardson and Hogan¹ demonstrated that rat dermatitis (acrodynia) is prevented or cured by an aqueous extract of yeast or rice bran, and by certain oils. The activity of the aqueous extracts was ascribed to the presence of a vitamin, presumably the one later designated as vitamin B₆. Since this communication is not immediately concerned with oils our observations, and those of other workers,² on their protective action will not be described. Birch³ reported that two factors are required to prevent or cure acrodynia. One is vitamin B₆, the other is one of the essential unsaturated fatty acids. György and Eckhardt⁴ obtained complete protection with a

* Contribution from the Department of Agricultural Chemistry, Missouri Agricultural Experiment Station Journal Series No. 680.

¹ Richardson, L. R., and Hogan, A. G., *Mo. Agr. Exp. Sta. Res. Bul.*, 1936, No. 241.

² Salmon, W. D., and Goodman, J. G., *J. Nutr.*, 1937, **13**, 477; Schneider, H., Steenbock, H., and Platz, Blanche R., *J. Biol. Chem.*, 1940, **132**, 539.

³ Birch, T. W., *J. Biol. Chem.*, 1938, **124**, 775.

⁴ György, P., and Eckhardt, R. E., *Nature*, 1939, **144**, 512.

combination of vitamin B₆ and various filtrates after fuller's earth adsorption. Under our experimental conditions both pantothenic acid and vitamin B₆ are required to cure this type of rat dermatitis, but additional time will be required to determine whether the cure is permanent.

The rats receive a ration of casein 20, sucrose 71, cellulose 3, cod liver oil 2, and salts 4, supplemented with 0.2 mg thiamin and 0.4 mg riboflavin per 100 g of food. After mild dermatitis developed vitamin B₆ was supplied to Group I, pantothenic acid to Group II, and both were supplied to Group III. Group IV contained three rats that had become moribund on vitamin B₆ and were then rescued

TABLE I.
A Combination of Pantothenic Acid and Vitamin B₆ Cures Rat Dermatitis.

No. of rats	Supplement	μg	Avg Exp. period, days	Avg wt		Remarks
				Initial, g	Final, g	
I						
5	B ₆	10	20.5	47	54	All improved, 4 had recurrence, died. 1 normal last observation.
4	B ₆	20	13	39	42	All improved, 3 had recurrence, 2 died. 1 normal last observation.
2	B ₆	30	18	40	37	Improved slightly, died.
II						
7	Sodium pantothenate*	70	9.3	40	34	2 healed. 1 died though norm. in appearance, 1 recurrence. 1 improved but died before it was normal. 4 died without improving.
3	Calcium pantothenate†	70	12	39	45	All improved but not normal. 2 had recurrence at last observation.
III						
2	B ₆ 10 + Na pantothenate	70	30	50	116	Dermatitis norm. disappeared,
3	B ₆ 20 + Ca pantothenate	70	12	44	72	Dermatitis norm. disappeared,
IV						
2	Na pantothenate	70	16	52	90	Dermatitis norm. disappeared,
1	" "	70	15	60	67	Dermatitis disappeared, but infection in both eyes, died.

*We are greatly indebted to Dr. T. H. Jukes who supplied us with this preparation. The amount is calculated from his assay in chick units.

†We are greatly indebted to Merck and Co., Rahway, New Jersey, who supplied us with this preparation.

with sodium pantothenate. Our observations are summarized in Table I.

All rats that received vitamin B₆ improved, but 9 of them had a recurrence within an average of 15 days. Of the other 2, one has been receiving vitamin B₆ for 12, the other for 30 days. Of the rats that received a pantothenic acid salt, 2 were cured. One of these was normal at death and the other had a mild recurrence of the dermatitis at the last observation. Some of the others improved but as yet none has made a complete recovery. When both vitamins were supplied simultaneously the animals recovered within a week and made considerable gains in weight. Three other rats, which failed to recover on vitamin B₆ alone and were on the point of death, responded in a similar manner when a pantothenic acid salt was also supplied. One animal died after the dermatitis had disappeared, from a secondary infection in both eyes.

The eyes of the animals which do not respond to vitamin B₆ alone are affected more severely than those of the animals which do not respond to pantothenic acid salts alone. The lids adhere and are soon covered by a large scab. If pantothenic acid is supplied in addition to vitamin B₆ at this stage the scab falls off, leaving a spectacled appearance which disappears without any additional treatment as the hair grows back in the denuded areas. Up to the present the other lesions characteristic of this type of dermatitis, if either vitamin B₆ or a pantothenate is supplied singly, are indistinguishable.

11536

Potencies of Vitamin K₁ and of 2-Methyl-1, 4-Naphthoquinone.

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In a previous investigation¹ we have confirmed the report of the marked antihemorrhagic activity of 2-methyl-1,4-naphthoquinone.² A careful comparison of the potencies of this compound and of pure vitamin K₁ by our 18-hour procedure³ showed that the latter is

* We wish to acknowledge financial assistance from the Theelin Fund administered by the Committee on Grants for Research of St. Louis University.

approximately one-half as active as the former. In view of this observation and the report that by the 6-hour procedure vitamin K₁ is only 1/30 as potent as 2-methyl-1,4-naphthoquinone,⁴ it seems desirable to publish the results that we have obtained in a comparison of the potencies of the two compounds by the 6-hour observation period.

Experimental. In our experiments, we have used for the evalua-

TABLE I.
Bioassay of Vitamin K₁ and 2-Methyl-1,4-Naphthoquinone.

Exp. No.	Compound	Dosage μ g	Chicks used,* No.	Response		
				Clotting time		Prothrombin time, Mean; S.E. sec
				<10 min %	Mean; S.E. min	
1	Vitamin K ₁ †	0.50	9	11	71.0 \pm 24.2	
		1.00	10	30	23.0 \pm 4.6	
	2-Methyl‡	0.50	14	50	13.1 \pm 2.6	
	Controls	none	8	0	>180.	
2	Vitamin K ₁	2.00	10	90	7.3 \pm 1.5	
		4.00	9	100	4.5 \pm 0.46	
	2-Methyl	0.50	9	67	17.2 \pm 4.5	
		1.00	9	100	5.3 \pm 0.90	
	Controls	none	9	0	>180.	
3	Vitamin K ₁	1.50	14	86	6.0 \pm 1.8	
	2-Methyl	0.50	10	50	12.1 \pm 2.1	
	Controls	none	10	0	110.	
4	Vitamin K ₁	2.00	19	100	4.5 \pm 0.46	
	2-Methyl	1.00	20	100	5.2 \pm 0.83	
	Controls	none	10	0	100.	
5	Vitamin K ₁	1.00	20	75	8.0 \pm 0.8	46.6 \pm 2.4
	2-Methyl	0.25	15	40	25.6 \pm 8.2	76.6 \pm 4.3
		0.50	30	70	12.0 \pm 2.7	47.0 \pm 3.0
	Controls	none	10	0	325.	93.3
6	Vitamin K ₁	2.00	15	100	5.8 \pm 0.5	28.7 \pm 1.2
	2-Methyl	1.00	15	100	5.5 \pm 0.4	33.7 \pm 0.8
	Controls	none	5	0	361.	99.0

*Chicks used in Exp. 4 were 15 days of age; all others 21.

†Natural vitamin K₁ was used in these experiments.

‡Used in this table as an abbreviation for 2-methyl-1,4-naphthoquinone.

¹ Thayer, S. A., Binkley, S. B., MacCorquodale, D. W., Doisy, E. A., Emmett, A. D., Brown, R. A., and Bird, O. D., *J. Am. Chem. Soc.*, 1939, **61**, 2563.

² Ansbacher, S., and Fernholz, E., *J. Am. Chem. Soc.*, 1939, **61**, 1924.

³ Thayer, S. A., McKee, R. W., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 194.

⁴ Ansbacher, S., Fernholz, E., and MacPhillany, H. B., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 655; Ansbacher, S., Fernholz, E., and Dolliver, M. A., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 652.

tion of the response of the chicks: (1) the percentage of chicks showing a clotting time of less than 10 minutes;⁵ (2) the mean clotting time; (3) the mean prothrombin time.⁵ Following Ansbacher's suggestion, a solution of the compound in cod liver oil was administered and the blood drawn 6 hours later for the evaluation of the reaction. Each assay included the response of the same lot of deficient chicks to the administration of one or 2 dosages of each compound and the mean clotting time of a control group. The data are summarized in the table.

In Experiments 1, 2, 3 and 4 the volume of cod liver oil used for administration of the compounds was 0.10 cc; in Experiments 5 and 6 only 0.05 cc was used. From other reports and the data of this paper it appears likely that in experiments in which the response is restricted to a period of 6 hours or less the volume of oil used may play a rôle in the absorption of vitamin K_1 and therefore in the apparent potency. We believe that the first 4 experiments of the table (0.10 cc oil used) indicate clearly that vitamin K_1 is approximately one-third as potent as 2-methyl-1,4-naphthoquinone, whereas the more complete data of Experiments 5 and 6 in which only 0.05 cc of solvent was used show that the vitamin is at least one-half as active.

Since the relative inactivity of vitamin K_1 with respect to 2-methyl-1,4-naphthoquinone and a purified extract of alfalfa is one of the important points in the claim that an antihemorrhagic compound more active than vitamin K_1 is present in alfalfa the discrepancy between Ansbacher's data and our observations should be examined. In his recent report Ansbacher⁴ gives for the minimal effective dose of 2-methyl-1,4-naphthoquinone 0.5 μ g and for vitamin K_1 15 μ g. The table in this paper shows that in the 4 experiments in which 0.50 μ g of 2-methyl-1,4-naphthoquinone was administered the percentages of the groups showing clotting times of less than 10 minutes were 50, 67, 50 and 90; with 1.0 μ g the response was always 100%. Consequently, it appears that the agreement with Ansbacher's data in the case of this compound is entirely satisfactory and that the discrepancy is due to the difference in the results obtained with vitamin K_1 . In our experiments the response to quantities of from 1 to 2 μ g ranged from 30 to 100%. The highest ratio of potencies of the two compounds (Exp. 2) was about 3:1; the lowest (Exp. 5) less than 2:1.

Since the purity of the vitamin K_1 used in these experiments is an

⁵ Almquist, H. J., and Klose, A. A., *Biochem. J.*, 1939, **33**, 1055.

important point, we wish to state that it was prepared by hydrolysis of the pure diacetyl dihydrovitamin K₁. The vitamin K₁ was purified by recrystallization at -70°C .

Analysis: Found C 82.39%; H 10.37%

Calculated C 82.62%; H 10.26%

$E_{1\text{ cm}}^{1\%}$ of a hexane solution at λ 249 m μ = 448

11537

Effect of Leukotaxine on Cellular Permeability and on Cleavage Development.

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Previous studies have demonstrated the presence of a nitrogenous substance in inflammatory exudates capable *per se* of increasing capillary permeability and of inducing rapid diapedesis of polymorphonuclear leukocytes. This substance has been named leukotaxine.¹⁻³ Its isolation has offered a reasonable explanation for two of the basic mechanisms in the development of the inflammatory reaction. Furthermore, this substance has been shown to possess none of the manifest physiological properties of histamine, thus rendering it difficult to accept the view that the latter plays a primary rôle in increasing capillary permeability at the site of injury.⁴⁻⁵ Kaiser has recently reached in regard to histamine an essentially similar conclusion, *i. e.*, at least as far as a long-standing inflammatory reaction is concerned.⁶

The present series of experiments have been undertaken in an endeavor to determine whether leukotaxine exerts any direct effect

* Aided by grants from the Milton Fund of Harvard University, from the International Cancer Foundation, and the Dazian Fund for Medical Research.

¹ Menkin, V., *J. Exp. Med.*, 1936, **64**, 485.

² Menkin, V., *J. Exp. Med.*, 1938, **67**, 129.

³ Menkin, V., *J. Exp. Med.*, 1938, **67**, 145.

⁴ Menkin, V., *Physiol. Rev.*, 1938, **18**, 366; *Dynamics of inflammation*, 1940, Macmillan Co., New York.

⁵ Menkin, V., and Kadish, M. A., *Am. J. Physiol.*, 1938, **124**, 524; Menkin, V., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 103.

⁶ Kaiser, P., *Schweiz. Z. f. allg. Path. u. Bakteriöl.*, 1939, **2**, 1.

on cellular permeability. The studies of Lucké and McCutcheon have demonstrated the usefulness of marine ova as effective material in the study of the living cell in its relation to permeability to water.⁷ The rate of passage of water placed in ova in a hypotonic medium is computed from the rate of change of volume. The permeability is derived from the equation: $\text{Permeability} = \frac{d v}{d t} / S (P - P_{\text{ex}})$ For

a detailed description of the terms involved in the equation, the reader is referred to the various publications of Lucké and McCutcheon.⁷⁻⁸

The present studies were made on the eggs of the sea urchin, *Arbacia punctulata*. For each experiment the ova were obtained from a single specimen of *Arbacia* and placed in sea water. The diameter of a number of ova was determined with an eyepiece micrometer. The cells are spherical and therefore the diameters were readily converted into measurements of surface area and volume. The ova were then transferred to hypotonic sea water (50%), and the rate of change of volume measured from minute to minute for a period of about 6 minutes. A smooth curve was drawn from which at a given time (2 to 3 minutes) the slope of the curve was obtained by drawing a tangent. Calculation of the permeability to water was then readily computed by applying the above equation. Ova were also exposed for several minutes to sea water containing leukotaxine in concentration of about 5 mg per cubic centimeter. These ova were then transferred to hypotonic sea water (50%) and their rate of swelling immediately measured. In each case the mean volume of several ova was plotted against time. Leukotaxine suspended in sea water induced a slightly acid medium which, after a prolonged interval, seemed to inhibit any change in the permeability of ova. The same type of result was obtained when normal ova were immersed in sea water previously acidified with HCl. For this reason, several experiments were performed by adjusting to a slightly alkaline level with 0.5 N NaOH the pH of the sea water containing leukotaxine. In experiments of short duration, this precaution was found superfluous. It seemed, in brief, as if only ova exposed for several hours to an acid medium failed to swell when subsequently transferred to a hypotonic medium. The data are summarized in Table I. It is clear that leukotaxine-treated ova showed, when immersed in a hypotonic medium, a considerable aug-

⁷ Lucké, B., and McCutcheon, M., *Physiol. Rev.*, 1932, **12**, 68.

⁸ Lucké, B., and McCutcheon, M., *Arch. Path.*, 1930, **10**, 662.

TABLE I.
Effect of Leukotaxine on Permeability of Ova to Water When Exposed to Hypo-
tonic Sea Water.

No. of experiment	Permeability of control ova*	Permeability of leukotaxine-treated ova*	% increase
1	.12	.22	83.3
2	.09	.16	77.7
3	.15	.25	66.6
4	.11	.15	36.4
5	.13	.16	23.1
Avg	.12	.19	57.4

*The units of permeability are in terms of cubic micra of water entering per minute, per square micron of cell surface, per atmosphere of pressure.

mentation in their permeability to water. The average increase over that of control ova was about 57%.

The present observations add further support to the view that probably leukotaxine increases capillary permeability by a direct effect on the permeability of the endothelial cell. Bier and Rocha e Silva originally postulated that histamine and leukotaxine were identical substances.⁹ Their view was severely criticized by the writer.^{4, 5} In view of the mass of accumulated evidence Bier has recently retracted his original contention¹⁰ (and personal communication, 1939). His present interpretation, however, that leukotaxine possibly liberates histamine which in turn is responsible for the increased capillary permeability¹⁰ is not supported by any observations. On the contrary, a concentration of histamine (1:20,000 to 1:50,000) equal to that recovered from exudates by Bier and Rocha e Silva, fails to induce the exact pattern of reaction on capillary permeability as elicited by either the untreated exudate or by leukotaxine recovered from such exudative material. It is also of interest to note that histamine fails to augment the permeability of sea urchin ova to water (Lucké, personal communication). In view of the opposite effect obtained with leukotaxine, it would be difficult to postulate that the latter acts on these cells by first releasing histamine. Finally, Ivy has recently succeeded in showing that whereas histamine induces increased gastric secretion of free acid in a Pavlov-pouch dog, leukotaxine is wholly ineffective in inducing any such effect (personal communication). This evidence supports further the view that leukotaxine fails to induce a release of

⁹ Bier, O., and Rocha e Silva, M., *Arqu. d. Inst. Biologico*, 1938, **9**, 109, 123, 129.

¹⁰ Bier, O., *Proc. Third International Congress for Microbiol.*, New York, 1940, p. 768.

histamine, since the latter in concentration as low as 0.1 mg manifests demonstrable secretion of free acid.

Lucké and McCutcheon showed that *Arbacia* ova placed in a hypotonic solution display an increased permeability to water. When these cells were returned to ordinary sea water and inseminated, cleavage frequently failed to occur or was atypical.⁸ These investigators expressed the belief that increased permeability is an expression of injury due to the rapid entrance of water. Leukotaxine not only enhances the permeability of sea urchin eggs to water but it also definitely disturbs cleavage development. This would suggest that this substance probably induces a certain amount of cellular injury.

The eggs of *Arbacia punctulata* were exposed to leukotaxine suspended in sea water in concentration of about 0.7 mg per cubic centimeter. The length of exposure to leukotaxine, prior to insemination in ordinary sea water, varied from one minute to 3 hours. In some instances, the pH of the sea water containing the leukotaxine-treated ova was adjusted approximately to that of ordinary

TABLE II.
Effect of Leukotaxine on Cleavage of *Arbacia* Ova.

Exp. No.	Experimental Time exposure to leukotaxine		Control Time exposure to sea water prior to fertilization	
	1-30 min % ova in cleavage	1-3 hr % ova in cleavage	1-30 min % ova in cleavage	1-3 hr % ova in cleavage
1	42 0	0 0	40	14
2	58 92	94 88 58	92	98
3	32	26 37 6	100	74 76
4	60	98 66 92	100	100 98
5	44 58	32	100	94
6	44 34 18 22	—	100	—
Avg	42	49.75	88.67	79.14

sea water by the addition of 0.5 N NaOH. This precaution induced essentially no difference in the ultimate results. The effect on cleavage formation obtained in leukotaxine-treated and in control ova is summarized in Table II. In both groups similar containers with approximately the same volume of suspension of eggs were utilized. It is clear that with the exception of one experiment most of the ova in the control group displayed, within several hours, variable numbers of blastomeres. The percentage of ova in cleavage exposed 1 to 30 minutes to sea water prior to insemination averaged 88.67. When exposed for longer intervals (1 to 3 hours) the percentage of ova in various stages of cleavage was slightly reduced, namely 79.14. These two figures stand in sharp contrast with the effect obtained in the leukotaxine-treated group. The number of ova with blastomeres averaged, in the experimental ova, 42 and 49.75% respectively. In other words, about half of the eggs exposed to leukotaxine failed to divide when subsequently inseminated.

Furthermore, the cleavage pattern in the majority of leukotaxine-treated ova appeared abnormal, being characterized by fewer blastomeres than in the control eggs and by unequal forms of division. This is exemplified in Fig. 1 and 2. The former shows normal development of an ovum about one hour and a half following insemination. Fig. 2 illustrates the type of development encountered in an ovum exposed to leukotaxine prior to insemination. The ovum is from the same specimen of *Arbacia* as the one in Fig. 1 and the intervals following insemination are approximately identical in both



FIG. 1.



FIG. 2.

cases. The illustrations represent histological sections after fixation in Bouin and acetic acid followed by 70% alcohol. This abnormal form of development in the leukotaxine-treated ova was frequently traced back to the fertilization reaction. The fertilization membrane appeared as a zone distinctly narrower than under normal circumstances. It was frequently found surrounded by sperms adhering to it (Fig. 2). Evidence of either cytolysis, absence or localization of the pigment in one area of the ovum were not of infrequent occurrence. These facts suggest that leukotaxine induces some degree of injury to the ova. It is also important to note that besides unequal cleavage, the leukotaxine-treated eggs displayed, after a given interval of time, considerably fewer blastomeres than in the untreated ova (Fig. 1 and 2). This strongly suggests that leukotaxine tends to retard the rate of cleavage.

Finally, sperms exposed for only a few minutes to leukotaxine induced fertilization of the ova of *Arbacia* and the subsequent cleavage pattern appeared to be unaltered. When, however, sperms were placed in contact with leukotaxine for about one hour, there was a sharp reduction in their fertilizing capacity. In some cases 98% of the ova failed to segment. This indicates that leukotaxine is evidently likewise injurious to sperms provided the latter are exposed to this substance for a sufficiently long interval.

Conclusions. Leukotaxine, the substance obtained from inflammatory exudates which is capable *per se* of increasing capillary permeability and of inducing leukocytic migration, markedly augments the permeability of sea urchin ova to water. Furthermore, a considerable number of ova exposed to this substance manifest abnormal cleavage development following their insemination. This appears in the form both of unequal cleavage and of an appreciable retardation in the rate of cell division. Sperms are also inactivated after prolonged exposure to leukotaxine. These various manifestations indicate that leukotaxine induces a certain degree of cellular injury when in contact with the ova or sperms of *Arbacia punctulata*. These effects on invertebrate eggs coupled with its rôle in inflammation suggest that leukotaxine may prove of biological significance in the study of cell division and permeability.

My thanks are due to Doctor B. Lucké for generous advice during the course of this study.

Protecting Action of Chemicals Related to Procaine on Ventricular Fibrillation During Cyclopropane Anesthesia.

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In a previous presentation¹ it was reported that procaine reduced the incidence of ventricular fibrillation following the intravenous injection of small doses of epinephrine into dogs during cyclopropane anesthesia. Since the injection of procaine solution into the circulation of man is frequently followed by untoward reactions, it seemed desirable to investigate the action of less toxic substances of the same chemical group. Para-amino benzoic acid, Paramon* and sodium para-amino benzoate were the drugs studied.

Fifty experiments were performed on 21 dogs. Preanesthetic medication, morphine sulphate one mg per kilo and scopolamine hydrobromide 0.04 mg per kilo was injected subcutaneously one hour before each experiment. The carbon dioxide absorption technic was utilized for cyclopropane anesthesia. An unobstructed airway was assured by an endotracheal tube fitted with an inflatable cuff. Depth of anesthesia was maintained at second plane as evidenced by the loss of the lid reflex and maintenance of intercostal activity. Electrocardiograms (lead II) were taken before, during and after drug administration.

The test injection of epinephrine was 0.01 mg per kilo in 5 cc of normal saline, given intravenously at the rate of 1 cc per 10 seconds. Para-amino benzoic acid and Paramon were administered intravenously at the dose of 5 to 10 mg per kilo in 20 cc of normal saline injected at the rate of 5 cc in 10 seconds. Sodium p-amino benzoate was administered at the dose of 10 to 40 mg per kilo in 5 cc of normal saline at the rate of 1 cc in 10 seconds.

The effects of p-amino benzoic acid were studied in 13 experiments on 5 dogs. Administration of this drug during cyclopropane anesthesia prior to the injection of epinephrine showed a protecting action against the production of cardiac irregularities. When it

¹ Burstein, Charles L., and Marangoni, Bruno A., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **43**, 210.

* Paramon is the calcium double salt of benzyl succinic and p-amino benzoic acids prepared and supplied by the Seydel Chemical Company.

was omitted, 4 out of 5 dogs died following ventricular fibrillation when epinephrine was injected during second plane cyclopropane anesthesia. Two test doses of epinephrine had been used in 2 of the cases and only one test dose in the other 2. All 4 of these animals had recovered from previous experiments in which they had been treated with p-amino benzoic acid prior to the injection of the same doses of epinephrine. In the animals that survived the administration of epinephrine alone, the cardiac irregularities were more severe than when the epinephrine injection was preceded by p-amino benzoic acid. One animal in this group developed ventricular fibrillation following the injection of one test dose of epinephrine preceded by p-amino benzoic acid. This animal, however, showed such marked emotional agitation prior to being anesthetized as to suggest the possibility of excess epinephrine secretion being an additive factor to the epinephrine administration.

The action of Paramon was studied in 21 experiments on 9 dogs. The results were similar to those obtained with p-amino benzoic acid. Noteworthy is the fact that when ventricular fibrillation developed in 2 of the animals following the injection of epinephrine alone, the intracardiac injection of 100 mg of procaine in 5 cc saline caused a change from ventricular fibrillation to auricular tachycardia and finally full recovery to sinus rhythm. Subsequently, the intracardiac injection of Paramon under the same conditions in the same animals was ineffective.

Sodium p-amino benzoate which is more soluble than the other 2 drugs permitted the use of larger quantities in less volume of solution. Doses of 10 to 40 mg per kilo were used and found to have effects similar to those of the other 2 dogs. Sixteen experiments on 7 dogs were performed with this drug. Five of the animals showed complete absence of cardiac irregularities when sodium p-amino benzoate was injected before one test dose of epinephrine whereas omission of sodium p-amino-benzoate resulted in ventricular fibrillation in 3 of the animals and ventricular tachycardia in the other 2. The remaining animals showed a few ventricular premature systoles when sodium p-amino benzoate was employed prior to epinephrine in contrast to the development of ventricular tachycardia in one case and ventricular fibrillation in the other when epinephrine alone was administered. In this group also, 2 of the animals that developed ventricular fibrillation were successfully treated by the intracardiac injection of procaine but succumbed when sodium p-amino benzoate was employed at the time of fibrillation.

Conclusions. The administration of p-amino benzoic acid, the calcium double salt of benzyl succinic and p-amino benzoic acids, or sodium p-amino benzoate prior to a test dose of epinephrine during cyclopropane anesthesia reduced the incidence of ventricular fibrillation. The intracardiac injection of procaine at the time when ventricular fibrillation developed effected a return to normal in a number of cases. Ventricular fibrillation was not ameliorated by the intracardiac injection of the other three p-amino benzoic acid derivatives.

The authors wish to express their appreciation for the helpful suggestions of Dr. Arthur C. DeGraff.

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**Anti-Catalase Activity of Sulfanilamide and Related Compounds.
VI. Further Studies on Sulfonhydroxamides.**

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In previous studies on the anti-enzymatic concept of the mode of action of sulfanilamide,¹⁻⁴ attention has been focused on catalase as one of the enzymes of importance. A time factor was postulated for the conversion of the inactive sulfanilamide to an active anti-catalase, which was presumed to result through oxidation to the hydroxylamino derivative. This furnishes an explanation of the characteristic lag period preliminary to the bacteriostatic action of sulfanilamide. It was therefore expected that p-hydroxylamino sulfanilamide, or a similar substance, would exert a bacteriostatic effect without this period of lag and that in addition the action would be more intensive. The sulfonhydroxamides contain a hydroxylamino group which, although located differently in the molecule, contributes anti-catalase activity. Hence they might

¹ Main, E. R., Shinn, L. E., and Mellon, R. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, **39**, 272.

² Shinn, L. E., Main, E. R., and Mellon, R. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, **39**, 591.

³ Main, E. R., Shinn, L. E., and Mellon, R. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **42**, 115.

⁴ Mellon, R. R., Locke, A. P., and Shinn, L. E., *Publication No. 11, A. A. A. S.*, 1939, pp. 98-113.

behave as preformed active derivatives. It was demonstrated⁵ that *p*-caproylamino benzenesulfonhydroxamide was capable of initiating bacteriostasis in broth cultures of the pneumococcus without the degree of lag manifested by sulfanilamide and that the bacteriostatic power per mole was more than 4 times as great as that of sulfanilamide. All of the sulfonhydroxamides examined were capable of effecting the accumulation of hydrogen peroxide in pneumococcus cultures.

These results were obtained in the absence of blood. The known reactivity of hydroxylamine and its derivatives for hemoglobin would lead to the expectation that in the presence of blood the hydroxylamino group would be destroyed before reaching the bacterial cell* with a resulting diminution of the bacteriostatic effect. It would not be expected that the immediate nature of the action would be impaired. This bacteriostasis should, however, prove to be transient. There are thus 3 points which can be tested experimentally.

Cultures of a virulent pneumococcus (Neufeld Type I) were established by seeding 1.5 cc samples of defibrinated rabbit blood contained in 1x11 cm tubes with 0.1 cc of a 1/100,000 broth dilution of a 16-hr. blood broth culture (300 organisms) and incubating at 39.5°C for 1.5 hr with intermittent shaking. Plate counts were made at the end of this period and the required compounds added as 0.1 cc of a blood dilution of an alcoholic stock solution. Controls received corresponding amounts of alcohol.† Incubation and shaking were continued and further counts made at 0.75, 1.75, 3.5, and 8.5 hr after the additions. Inhibition was calculated as in the preceding paper.⁵

Fig. 1 A, B, C, F, gives the results of such experiments for *p*-caproylamino benzenesulfonhydroxamide and sulfanilamide. In order to attain a degree of bacteriostasis comparable with that pro-

⁵ Main, E. R., Shinn, L. E., and Mellon, R. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **43**, 593.

* Blood containing the sulfonhydroxamides took on a brownish hue during incubation. While no experimental proof of the point is available, this was in all probability the result of methemoglobin formation and an indication of the lability of the hydroxamide group. It has been found in the Sharp and Dohme Laboratories that these sulfonhydroxamides decompose in contact with moisture to yield oxides of nitrogen. Obviously these oxides would tend to produce methemoglobin. They also appear to be responsible for the yellow color produced when broth containing the sulfonhydroxamides was treated with *o*-tolidine.⁵

† The concentration of alcohol in the cultures was 1-2%. It appeared to have no effect on growth.

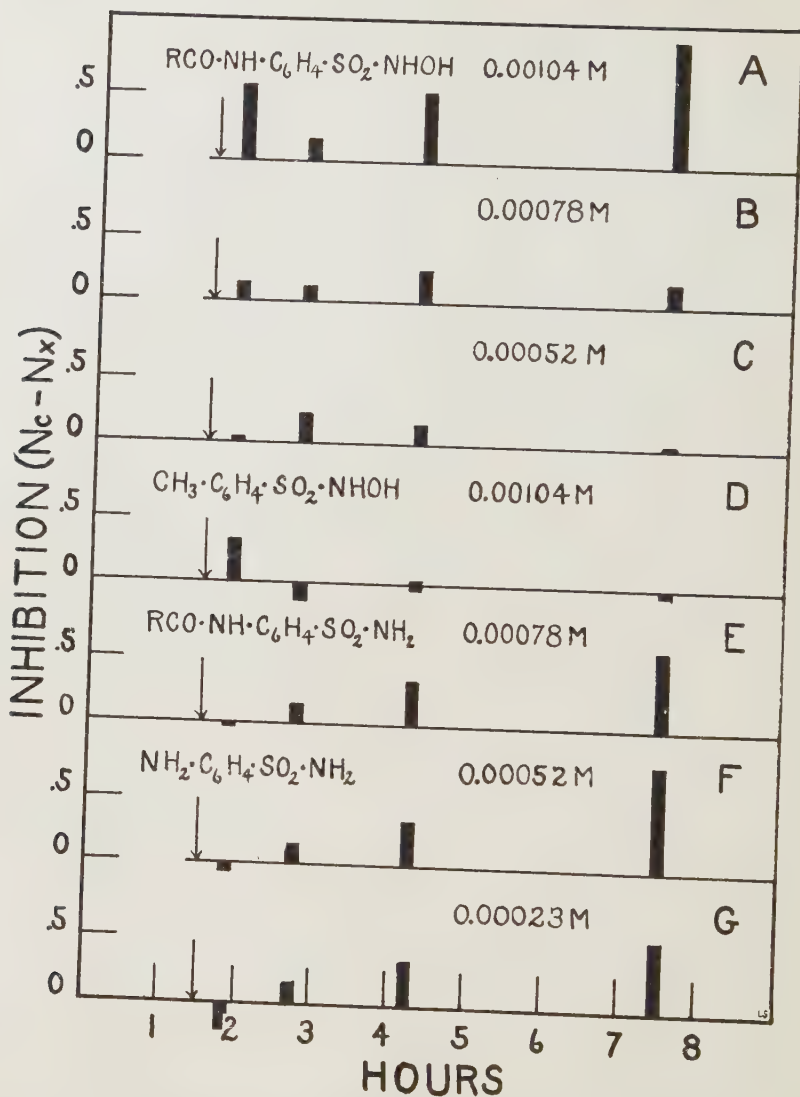


FIG. 1.

Inhibition of growth of Type I pneumococcus in defibrinated rabbit blood at 39.5°C by: (A) *p*-caproylaminobenzenesulfonhydroxamide,† 0.00104 M; (B) the same, 0.00078 M; (C) the same, 0.00052 M; (D) *p*-toluenesulfonhydroxamide,† 0.00104 M; (E) *p*-caproylaminobenzenesulfonamide,† 0.00078 M; (F) sulfanilamide, 0.00052 M; (G) the same, 0.00023 M. Inhibition is expressed as the difference between the number of generations produced per hour in a control culture (N_c) and in a culture containing the compound added (N_x) over the intervals 1.5-2.25, 2.25-3.25, 3.25-5.0, and 5.0-10.0 hr. The values are plotted at the mid-point of the interval concerned. Cultures were inoculated at 0 hours and the compounds added at 1.5 hr (arrow).

† Furnished us through the courtesy of Dr. Maurice Moore of Sharp and Dohme, Inc.

duced by 0.00052 M (9 mg%) sulfanilamide, the concentration of the hydroxamide must be raised to 0.00104 M. This is in contrast to the results for the same compound in broth where less than 0.00016 M was required for the same result.⁵ The expected action of blood in reducing the bacteriostatic efficacy of this type of compound is hence demonstrated. As was anticipated, the bacteriostatic power of the sulfonhydroxamide is established within the first observational period, whereas that of sulfanilamide shows a brief period of stimulation.

The dip in bacteriostatic power at the second interval in Fig. 1 A suggests two diverse actions. The second action appears at about the time that the first is vanishing and resembles that produced by sulfanilamide. This suggests that it can be attributed to a free *p*-amino group produced from the caproylamino group whereas the transient portion can be referred to the hydroxamide group.

This was tested by employing two other compounds: *p*-toluenesulfonhydroxamide and *p*-caproylamino benzenesulfonamide in which the two groups are removed to separate molecules. The results are shown in Fig. 1 D and E. When the caproylamino group is absent (D) only the immediate transient action is obtained. When the hydroxamide group is replaced by an amide group (E) no immediate action is found but the second action of *p*-caproylamino benzenesulfonhydroxamide is approximately reproduced. Hence it appears that the postulate regarding the prompt but transient nature of the action of the hydroxamide group is correct but that the complete acylaminobenzenesulfonhydroxamide molecule has a double activity. This result made it advisable to investigate the extent of deacylation of caproylamino derivatives in blood.

To whole defibrinated rabbit blood was added *p*-caproylamino benzenesulfonhydroxamide or *p*-caproylamino benzenesulfonamide to a concentration of 0.00078 M. The samples were incubated at 39.5° and portions withdrawn for analysis by the Marshall method. The results are given in Table I. Both compounds underwent substantial deacylation in blood. In broth no significant deacylation of the sulfonhydroxamide was noted; indicating that deacylation in blood was largely enzymic. That the degree of conversion to free amine in the case of the sulfonhydroxamide is sufficient to account for the bacteriostasis produced is not conclusively demonstrated but in Fig. 1 G is shown the result of experiments with a lower concentration of sulfanilamide (4 mg%) in which very substantial bacteriostasis was produced.

TABLE I.
Deacylation of *p*-Caproylaminobenzenesulfonhydroxamide and *p*-Caproylaminobenzenesulfonamide in Broth and Defibrinated Rabbit Blood at 39.5°C.

Hr	A			B	
	% deacylation in broth pH 7.4	% deacylation in blood	mg% sulfanilamide equivalent in blood	% deacylation in blood	mg% sulfanilamide equivalent in blood
0	1.3	0.8	0.1	0.4	0.05
0.75	—	3.6	0.5	4.1	0.5
1.75	—	5.8	0.8	7.5	1.6
3.50	1.3	10.8	1.5	18.0	2.7
6.75	—	15.7	2.1	32.0	4.3
24.00	—	29.8	4.0	76.0	10.2

A. *p*-caproylaminobenzenesulfonhydroxamide 0.00078 M.

B. *p*-caproylaminobenzenesulfonamide 0.00078 M.

Discussion. *p*-Caproylaminobenzenesulfonhydroxamide appears to represent an approach to that hypothetical compound which shall be "preformed" in the sense of possessing the group or groups necessary to bacteriostatic activity which are normally formed by the micro-organism itself. That the present compound is not ideal is demonstrated by the transient nature of the powerful early effect. The demonstration of two successive activities arising from the same molecule opens interesting fields of speculation and experiment regarding future objectives in the production of better therapeutic agents.

Summary. *p*-Caproylaminobenzenesulfonhydroxamide produces bacteriostasis of pneumococci in blood without the lag characteristic of sulfanilamide. The activity is only about one-eighth of the corresponding bacteriostatic power in broth. That the immediate nature of the effect is due to the hydroxamide group was demonstrated by the use of *p*-toluenesulfonhydroxamide in which the potentially active *p*-amino group is absent. The effect of the hydroxamide group is transient in nature. A second period of bacteriostatic activity manifested by *p*-caproylaminobenzenesulfonhydroxamide is probably due to the free amino group formed by deacylation of the caproylamino group.

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Bioassay of Water-Soluble Antihemorrhagic Compounds by Intravenous Administration.*

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Since the natural vitamin K compounds are not soluble in water and in many cases superior therapy could be attained by intravenous administration, several different water-soluble antihemorrhagic compounds have been prepared. (Almquist and Klose;¹ Thayer, *et al.*,² Fieser and Fry;³ Foster, *et al.*,⁴ and Ansbacher, *et al.*⁵).

Supplementing our previous reports (Thayer, *et al.*),⁶ we have assayed 2-methyl-1,4-naphthoquinone and several closely related water-soluble compounds by different methods in order to determine their respective potencies. Originally our products for assay were administered orally but recently the realization that the potencies by parenteral administration might be very different has led us to employ intravenous injection. Compounds which are active orally might be inactive parenterally due to their avoidance of the enzymes of the gastro-intestinal tract or to their too rapid excretion by the kidneys.

The potencies have been estimated from a comparison of the effects of the compounds under investigation and 2-methyl-1,4-naphthoquinone on the, (1) mean prothrombin time, (2) mean clotting time, and, (3) percentage of positive responses.⁷ Each

* We wish to acknowledge financial assistance from the Theelin Fund administered by the Committee on Grants for Research of St. Louis University.

¹ Almquist, H. J., and Klose, A. A., *J. Am. Chem. Soc.*, 1939, **61**, 1611.

² Thayer, S. A., Binkley, S. B., MacCorquodale, D. W., Doisy, E. A., Emmett, A. D., Brown, R. A., and Bird, O. D., *J. Am. Chem. Soc.*, 1939, **61**, 2563; Doisy, E. A., MacCorquodale, D. W., Thayer, S. A., Binkley, S. B., and McKee, R. W., *Science*, 1939, **90**, 407.

³ Fieser, L. F., and Fry, E. W., *J. Am. Chem. Soc.*, 1940, **62**, 228.

⁴ Foster, R. H. K., Lee, J., and Solmssen, U. V., *J. Am. Chem. Soc.*, 1940, **62**, 453.

⁵ Ansbacher, S., Fernholz, E., and Dolliver, M. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **43**, 652.

⁶ Thayer, S. A., Cheney, L. C., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A., *J. Am. Chem. Soc.*, 1939, **61**, 1932.

⁷ Thayer, S. A., McKee, R. W., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 194.

TABLE I.
Bioassay of Water Soluble Compounds (Intravenous Administration)

Compounds	Dosage, μ g	Equivalent in μ g of standard, \ddagger μ g	Chicks used (21 days of age) No.	Response normal clotting time, %	Clotting time, min mean; S.E.,	Prothrombin time, sec mean; S.E.,
1,4-dihydroxy-2-methylnaphthalene monosuccinate	0.751 \ddagger	0.68	12	58	17.8 \pm 6.8	41.9 \pm 4.9
2-methyl-1,4-naphthoquinone (standard)	1.502	1.0	30	93	6.7 \pm 0.5	29.1 \pm 2.2
Controls lot 1	0.961		10	80	12.7 \pm 4.9	35.7 \pm 4.1
" " 2	1.432		20	100	6.0 \pm 0.8	28.6 \pm 1.4
	—		10	0	129.7	58.0
	—		10	0	109.2	60.6
di-potassium-1,4-dihydroxy-2- methylnaphthalene-disulfate	2.503	1.0	14	64	29.1 \pm 6.4	44.3 \pm 8.6
	6.006	2.3	15	86	7.0 \pm 0.82	33.5 \pm 0.48
2-methyl-1,4-naphthoquinone (standard)	6.005	2.3	14	71	8.9 \pm 1.1	37.1 \pm 1.50
Controls lot 3	0.963		10	60	9.9 \pm 5.6	28.6 \pm 1.2
	0.966		15	100	4.7 \pm 0.7	28.6 \pm 4.1
	—		10	0	155.0	61.2
4-amino-2-methyl-1-naphthol*	1.002	0.74	29	89	6.7 \pm 0.8	32.8 \pm 1.5
	1.501	1.1	15	93	7.0 \pm 1.4	27.8 \pm 1.2
2-methyl-1,4-naphthoquinone (standard)	1.504	1.0	15	93	7.1 \pm 0.9	30.3 \pm 3.6
Controls lot 4	1.434		20	100	6.1 \pm 0.90	27.4 \pm 1.6
	—		10	0	104.0	60.6
4-amino-3-methyl-1-naphthol*	1.305	1.0	15	93	5.7 \pm 0.65	32.5 \pm 1.2
4-amino-2-methyl-1-naphthol*	1.305	1.0	15	73	8.4 \pm 1.4	35.5 \pm 1.30
Controls lot 5	—		10	0	296.6	67.8
Normal chicks	—		10	100	4.3 \pm 0.1	26.5 \pm 1.0

* As the crystalline hydrochloride containing 0.5 M ethanol.

\ddagger The superscript designates the lot of chicks used for that assay.

\ddagger The values of column 3 were obtained by multiplying the values of column 2 by the ratio of the molecular weights of 2-methyl-1,4-naphthoquinone and the respective compound.

assay has been controlled by an accompanying assay of a standard substance (usually 2-methyl-1,4-naphthoquinone) on the same lot of deficient chicks to eliminate the variation in deficiency found in different groups of chicks.

The basal diet used in all of our experiments is the one described by Almquist.

Assays—The compounds were dissolved in 0.85% NaCl solution. The chicks were slightly anesthetized with ether and the solution was injected directly into the jugular vein of the chick (0.1 cc of solution). Bleeding was minimized by the use of a fine needle (27 gauge) and by entering the vein through the subcutaneous tissues. Eighteen hours after the injection blood was drawn from the brachial vein into a small tube (micro test tube of Fischer)⁸ and placed at once in a thermostat adjusted to 38.5-39.5°C and the time for coagulation determined. The chick's head was clipped off with scissors and 1.8 cc of blood collected in a vial containing 0.20 cc of 0.1 M sodium oxalate solution. The method of Almquist and Klose⁹ was used for determining prothrombin time.

Early in our work on Vitamin K, we realized that the variability of the degree of deficiency of different lots of chicks (see values for the controls Table I) could produce gross inaccuracies in the bioassay. Originally we standardized the deficiency of each lot of chicks by determining the response to 2 different dosages of a stock solution which had been obtained from alfalfa. Later,² we suggested the adoption of pure crystalline 2-methyl-1,4-naphthoquinone as the basic standard. The variability of the response of these lots of chicks (1, 3 and 6) to the administration of 0.96 μ g of 2-methyl-1, 4-naphthoquinone (Table I) emphasizes the necessity of ascertaining the response of each lot of chicks to a standard.

On a weight basis, 1,4-dihydroxy-2-methylnaphthalene monosuccinate, 4-amino-2-methyl-1-naphthol and 4-amino-3-methyl-1-naphthol administered intravenously are, perhaps, slightly less active than 2-methyl-1,4-naphthoquinone; on a molar basis all are fully as active. The potency of the disulfate on a weight basis is somewhat less than one-sixth and on a molecular basis approximately one-third that of 2-methyl-1,4-naphthoquinone.

Summary. Using intravenous administration it has been found that on a molecular basis the potencies of all the compounds with

⁸ Fischer, A., *Pflüger's Arch. ges. Physiol.*, 1930, **225**, 737.

⁹ Almquist, H. J., and Klose, A. A., *Biochem. J.*, 1939, **33**, 1055.

the exception of the disulfate are approximately equal to that of the standard, 2-methyl-1,4-naphthoquinone. The disulfate is about one-third as potent on a molecular basis, but owing to the much larger molecular weight its activity per milligram is somewhat less than one-sixth that of 2-methyl-1,4-naphthoquinone.

11541 P

Riboflavin Determinations on Normal Liver and Liver Tumor.

HERBERT KAHLER AND EVERETT F. DAVIS. (Introduced by Carl Voegtlin.)

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It was decided to use a fluorometric method in making quantitative estimations of riboflavin from fresh liver tissue after several checks had been run against the Snell and Strong method.* The fluorometric method here used is first to determine the total fluorescence, then eliminate the riboflavin by raising the alkalinity to pH 11 and determining the "interfering" fluorescence. The difference between these two values gives the approximate riboflavin value. The fluorescence was measured with a photocell using suitable optical filters.

Liver tumors from Osborn-Mendel rats which had been fed 2-amino 5-azo toluene for a long period were used. These animals were kindly put at our disposal by Dr. E. Emmart. Samples of liver which had been perfused with saline were ground with n/10 HCl, autoclaved for 15 min. and clarified by precipitation at pH 5-6, followed by filtering through No. 42 paper. Recovery of added riboflavin was about 97% by this fluorometric method.

Some of the material was given a short low temperature drying by the lyophile process eliminating most of the water. The data in the tables indicate that the difference between normal and tumor liver is not due to water content.

Comparisons were made between normal livers from normal rats, nontumor bearing liver from dye-fed rats, liver tumors from dye-fed rats, residual liver from which the tumor had been excised, fetal liver, and leg muscle. In the case of a general riboflavin deficiency it would be expected that lower riboflavin values for the muscle would be ob-

* Made by Drs. Isbell and Wooley of the National Institute of Health.

RIBOFLAVIN DETERMINATIONS ON LIVER AND LIVER TUMOR 605

TABLE I.
 Riboflavin Concentration in Micrograms per Gram of Fresh Tissue.

Rat No.	Histologic diagnosis on liver	γ/g		Leg muscle, γ/g
		Tumor	Liver residue	
1	Tumor (no diagnosis)	8	16	
2	" " "	12	15	
3	Carcinoma	11	15	
4	Pooled carcinoma, hepatoma	16	16	
5*	Carcinoma, Region {	A	15.7	2.52
		B	13.2	
		C	18.6	
6*	Hepatoma	19.2	20.7	
7	"	21.3	21.3	
8	"	16.9	24.7	
9	" , carcinoma	22.2	22.2	2.80
10	" , cirrhosis	25.7	29.8	2.95
11	" , carcinoma			
	+ cirrhosis	26.8	27.95	2.56
	Dye fed; no tumors	γ/g		
12	Cirrhosis	26.14		2.1
13	" (slight)	27.84		2.9
14	" "	29.8		3.3
15	"	31.04		
16	"	34.0		2.1
17	Essentially normal	35.7		
	Normal rats, normal diet			
18	Normal	25.0		
19		25.5		
20		25.85		
21		28.6		
22		30.8		
23		30.9		
24		39.0		
25	Normal fetal liver	4.7		
26	" " "	6.3		

*Large amount of blood in tissue.

TABLE II.
 Riboflavin Concentration in Micrograms per Gram of Dried Tissue.

Rat No.	Histology	γ/g dry wt	γ/g wet wt (calc.)
27, 28	Pooled material, carcinoma		
29, 30	and hepatoma	58.9	14.9
31	Pooled hepatoma, carcinoma and cirrhotic liver	59.4	15.0
32	Normal	106.6	29.0
33	"	106.8	29.0

tained. A value of 1.4 γ is reported¹ for adult rat muscle on a deficient diet.

¹ Fraser, H., Topping, N., and Isbell, H., *Public Health Rep.*, 1940, **55**, 280.

In the absence of any other criteria the estimation of "malignancy" was based solely on histologic grounds. The term hepatoma was used to denote growths which reproduced the normal liver structure so closely that there was no histologic indication of "malignancy". On the other hand certain of these tumors manifested such extreme degrees of deviation from normal liver cell structure and architecture that they were termed carcinomas to denote their malignant histologic appearance.

The following conclusions may be drawn from these results:

1. The average value for normal and cirrhotic livers of 30.1 γ (age 256 to 550 days) agrees with Fraser, Topping and Isbell's¹ values for considerably younger rats of 33 γ (age 130 days).

2. Continuously dye fed rats with cirrhosis not developing gross tumors gave normal riboflavin content for liver.

3. Livers in which tumors had developed gave a lower value per gram of fresh tumor tissue and per gram of residual tissue than normal liver.

4. On the basis of lyophilized dry weight normal liver is considerably higher in riboflavin concentration than tumor material.

5. The muscle values indicate that the animal is not suffering from a general riboflavin deficiency.

These results are consistent with the earlier findings² that lactic acid accumulates in various tumors especially following large sugar injections. According to current views³ pyruvic acid which is formed from sugar break down is normally oxidized to CO₂ and H₂O through molecular oxygen and the flavoprotein-cytochrome enzymes. With a deficient oxygen and/or enzyme concentration the pyruvic acid is converted to lactic acid giving tumors a low pH. In this connection see the recent finding of low coenzyme in tumor⁴ and the high ratio of reduced to oxidized cozymase in Jensen sarcoma.⁵

The histologic diagnoses were made by Drs. H. L. Stewart and Hugh G. Grady.

² Voegtlin, C., Fitch, R., Kahler, H., Johnson, J. M., and Thompson, J. W., *Nat. Inst. Health Bull.*, No. 164, 1935.

³ Eric Ball, *Johns Hopkins Hosp. Bull.*, 1939, **65**, 253.

⁴ Bernheim, F., and Felsovanyi, A. V., *Science*, 1940, **91**, 76.

⁵ Euler, H. V., Schlenk, F., Heiwinkel, H., and Högberg, B., *Z. f. physiol. Chem.*, 1938, **256**, 208.

Absorption of Water-Soluble Vitamin K from Intestinal Tract.*†

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Bile salts are known to be essential for the absorption of fat-soluble forms of vitamin K from the intestinal tract, but it is not known whether they are of benefit in the absorption of the water-soluble forms. To obtain data regarding this, we have used bile-obstructed rats in which the vitamin K reserves were depleted pre-operatively by the technic previously reported from this laboratory.¹ After ligation of the bile duct, the animals were placed on a diet² from which vitamin K was still more rigidly excluded. Within 3-4 days the prothrombin level falls into the bleeding zone. The subsequent rise in prothrombin, following the oral administration of vitamin K, was used as a measure of the extent to which the vitamin is utilized, either with or without supplements of bile salts.

Prothrombin determinations were made by the 2-stage technic of Warner, Brinkhous, and Smith;^{3,4} bile salts (1 cc of 3% sodium taurocholate) and vitamin K were given through a metal tube into the stomach. As a source of water-soluble vitamin K, we used the potassium salt of the disulfuric acid ester of 2-methyl-1,4-naphthohydroquinone. Since this work was initiated the sodium salt of this compound was described by Fieser.⁵ It is apparently somewhat less potent⁶ than 2-methyl-1,4-naphthoquinone, when the two

* Aided by a grant from the John and Mary R. Markle Foundation. Funds for assistance were also supplied by the Graduate College, State University of Iowa.

† The potassium salt of the disulfuric acid ester of 2-methyl-1,4-naphthohydroquinone, used in these experiments, was prepared in November, 1939, as a part of a cooperative program in which a series of compounds of this type was synthesized by George H. Coleman, J. J. Carnes and D. W. Kaiser of the Department of Chemistry, State University of Iowa.

¹ Flynn, Joseph E., and Warner, E. D., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **43**, 190.

² Tidrick, Robert T., Joyce, Frank T., and Smith, H. P., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **42**, 853.

³ Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

⁴ Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, **66**, 801.

⁵ Fieser, L. F., and Fry, E. M., *J. Am. Chem. Soc.*, 1940, **62**, 228.

⁶ Ansbacher, S., Fernholz, E., and Dolliver, M. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **43**, 652.

TABLE I.
Response of Rats* to a Water-soluble Form of Vitamin K.

Days of treatment	Prothrombin levels (% of normal)		
	2 γ of vitamin daily	5 γ of vitamin daily	8 γ of vitamin daily
Results with vitamin K plus bile salts.			
0	24	19	17
1	14	52	57
2	dead†	52	78
			106
Results with vitamin K but without bile salts.			
0	15	12	29
1	14	16	50
2	15	38	86
3		55	—
4		68	91

* Albino rats weighing 200 to 300 g.

† Autopsy showed extensive intraabdominal hemorrhage.

compounds are compared on a molar basis. Our own experience indicates that the compound is non-toxic when given orally to rats in doses 100 times the physiological requirements.

Results. Table I shows typical examples of the response of K-deficient rats following administration of daily doses of 2, 5 and 8 μ g of the water-soluble compound. It is readily seen that bile salt did not appreciably modify the therapeutic efficacy of the vitamin. At the level of 2 μ g, the prothrombin level remained in the bleeding zone. At the level of 5 μ g, the recovery was 35-70% complete, both with and without bile salt, and with daily doses of 8 μ g the prothrombin rose above the 75% level.

When fat-soluble forms of vitamin K are administered to man, the danger always exists that the dose of bile salt will not be adequate in amount, or that the bile salt may not mingle properly with the vitamin following dissolution of the capsules. The oral administration of water-soluble vitamin K, if effective in man, will eliminate this problem. It will also eliminate much of the nausea and vomiting produced by the bile supplements. It is obvious that when the acute phase of the disease subsides, bile salt should then be given to enable absorption of the many fat-soluble components of the diet.

Effect of Vitamin K on Hypoprothrombinemia of Experimental Liver Injury.*

K. M. BRINKHOUS AND E. D. WARNER.

From the Department of Pathology, State University of Iowa, Iowa City.

It has been shown that the plasma prothrombin level declines when the liver is injured^{1, 2, 3} or when it is excised, either in part⁴ or *in toto*.^{5, 6} Hypoprothrombinemia develops also with vitamin K deficiency. In man, the two factors are often combined and the response to vitamin K is commonly incomplete. In such patients, the extent to which vitamin K compensates for the liver injury, if at all, is difficult to determine. To study this question further, we have performed experiments to determine the influence of vitamin K in excess on the hypoprothrombinemia which develops following liver injury alone.

Liver injury was produced in dogs (10-15 kg) by repeated administration of small doses of chloroform, as described previously.² In each experiment, 2 dogs of the same weight were given identical diets (mixed table scraps) and identical doses of chloroform. In addition, one of the animals of each pair received a daily vitamin K supplement consisting of the petroleum ether extract of 200 g alfalfa meal, emulsified in 30 cc of 2% solution of Wilson's bile salt. Plasma prothrombin determinations were made by the method of Warner, Brinkhous and Smith.^{1, 2}

Almost identical results were obtained in each of the 4 experiments performed. Chart 1 shows a typical experiment. It is seen that the administration of vitamin K failed to modify in any way, either the fall in prothrombin with chloroform administration or the rise in prothrombin during the recovery period.

In another experiment the daily vitamin supplement was started

* Aided by a grant from the John and Mary R. Markle Foundation. Funds for technical assistance were supplied by the Graduate College, State University of Iowa.

¹ Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

² Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, **66**, 801.

³ Quick, A. J., *J. A. M. A.*, 1938, **110**, 1658.

⁴ Warner, E. D., *J. Exp. Med.*, 1938, **68**, 831.

⁵ Warren, R., and Rhoads, J. E., *Am. J. Med. Sci.*, 1939, **198**, 193.

⁶ Andrus, W. D., Lord, J. W., and Moore, R. A., *Surgery*, 1939, **6**, 899.

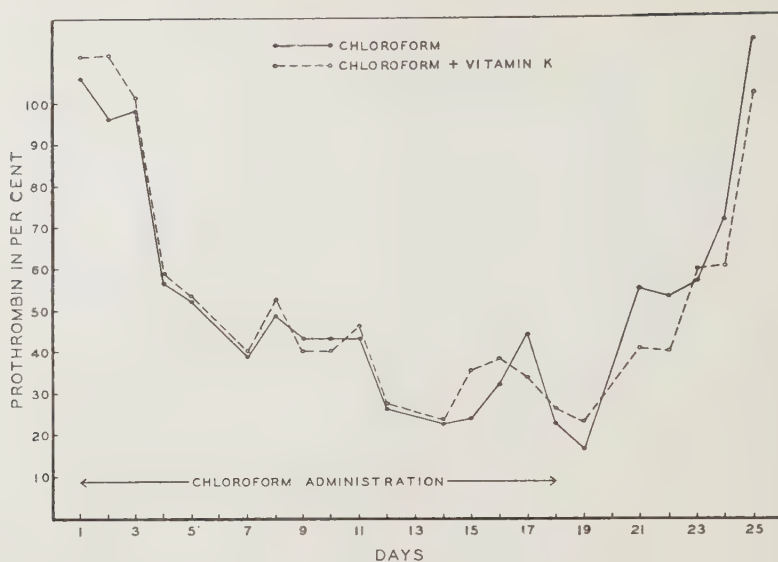


CHART 1.

The consecutive daily doses of chloroform given during the 18-day period of chloroform administration were 2, 2, 3, 2, 3, 2, 3, 3½, 3½, 4½, 5, 5, 4, 5, 6, 6, 7 and 6 cc.

one week prior to the beginning of chloroform administration. This procedure likewise had no demonstrable effect on the fall in plasma prothrombin.

It is suggested that when vitamin K deficiency and liver injury are both present, as in many patients, administration of the vitamin may correct the former, but one cannot expect that an excess of the vitamin will compensate for the element of liver injury.

Summary. The hypoprothrombinemia, which develops following liver injury (chronic chloroform intoxication), is not influenced by vitamin K administration.

11544 P

Study of Certain Tissue Lipids in Generalized Lipodystrophy
("Lipohistiodiaresis").*

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An unusual opportunity to study the lipid composition of various tissues in an extremely rare condition, that of generalized lipodystrophy, was offered when death occurred in a 9-year-old boy in whom an almost complete absence of adipose tissue from the body had been present for the past 6 years (Case 1). In addition to this remarkable apparent lack of body fat, the symptom complex was composed of cirrhosis of the liver, chronic fibrosis of the spleen, pancreas and certain lymph nodes, and diabetes mellitus. Necropsy was begun within one hour following demise, at which time samples of various tissues were obtained. After being weighed, the specimens (usually about 1 g of tissue) were placed in 95% alcohol and allowed to stand for 24 hours. The tissues were then ground with sea sand in a mortar, rinsed several times with alcohol and ether, returned to the original flasks, and sufficient ether added to make approximately a 3:1 alcohol-ether mixture. The flasks were immersed in a boiling water bath for about 5 minutes and allowed to cool; the contents were filtered through fat-free filter paper into volumetric flasks, brought to volume, and stored in a refrigerator until analyses were made. Aliquots were measured and the following procedures employed: The method of Wilson and Hansen¹ was used for the determination of the unsaponifiable and saponifiable fractions, while the technic followed by Hansen² was used for the determination of the fatty acids in the acetone-insoluble (phospholipid) fraction and the acetone-soluble (cholesterol ester-neutral fat) fraction. The total cholesterol and cholesterol esters were determined by the procedure described by Bloor,^{3, 4} the photoelectric colorimeter being used in obtaining the final readings. For the control studies, similar tissues from a 14-year-old boy dying in

* Aided by grants from Mead Johnson and Company and the Medical Graduate Research Fund of the University of Minnesota.

1 Wilson, Wm. R., and Hansen, Arild E., *J. Biol. Chem.*, 1936, **112**, 457.

2 Hansen, Arild E., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 376.

3 Bloor, W. R., *J. Biol. Chem.*, 1916, **24**, 227.

4 Bloor, W. R., and Knudson, A., *J. Biol. Chem.*, 1916, **27**, 107.

TABLE I.
Lipid Composition of Various Tissues in Child with Generalized Lipodystrophy
(Case 1—D.Z.) and in Child with Subacute Nephritis (Case 2—G.C.).

Lipid fraction	Perirenal		Subcutaneous	Liver		Skin	
	Case 1	Case 2	Case 2	Case 1	Case 2	Case 1	Case 2
Unsap. fraction*	459	902	694	1,197	615	306	316
Total fatty acids							
Sap.*	452	44,610	51,144	1,490	3,156	340	10,025
M.W.	292	—	279	294	290	276	274
I.N.	102	62	66	107	102	68	64
Acet. Sol. F. A.							
Sap.*	214	36,075	50,076	607	1,498	209	10,034
M.W.	268	274	278	293	281	—	274
I.N.	86	60	65	83	74	68	64
Acet. Insol. F.A.							
Sap.*	244	199	309	797	1,599	127	160
M.W.	294	—	—	301	296	—	—
I.N.	127	—	—	123	121	—	—
Cholesterol							
Total*	296	242	—	893	356	162	102
Ester*	83	194	—	329	77	29	32
Neutral Fat F. A.* (Calc.)	130	44,320	50,000	360	1,500	190	10,010

*Expressed in mg per 100 g wet tissue.

uremia from subacute nephritis (Case 2), the best available material at the time, were used.

The results are summarized in Table I.

It is readily apparent from inspection of the data in Table I that the total fatty acids of the various tissues are distinctly less in the child with the generalized lipodystrophy (Case 1) than in the control subject (Case 2). In the perirenal tissues, the unsaponifiable fraction and the cholesterol esters are also less in Case 1, while the values for the acetone-insoluble (phospholipid) fatty acids are essentially the same in both cases. As regards the subcutaneous material, it was impossible to find any adipose tissue in Case 1 which was suitable for analysis. In the liver, the value for the total fatty acids in Case 1 is but one-half that of Case 2, while the unsaponifiable fraction as well as the total cholesterol and cholesterol ester values are higher in this child with lipodystrophy. The acetone-insoluble (phospholipid) fatty acids in this tissue are definitely less in Case 1. As regards the skin, it is interesting to note that the values for the unsaponifiable fraction, total cholesterol, cholesterol esters, and the acetone-insoluble (phospholipid) fatty acids are essentially the same in both cases. On the other hand the

total fatty acid values of the skin in Case 1 are greatly diminished when compared with those of the control subject (Case 2). In spite of the great difference in the amount of fat present in this tissue, we find that the qualitative characteristics of the fatty acids as regards average molecular weight and average iodine number are practically the same in both instances.

That there is a definite lack of fatty acids in these tissues becomes even more striking when we consider the calculated values for the neutral fatty acids. These calculations disclose that the approximate values for the neutral fat fatty acids in the hepatic tissue of our control subject (Case 2) are 5 times that of those for the child with the generalized lipodystrophy, while those in the skin are 50 times that of those in Case 1. Even more striking is the finding that the tissues in the perirenal region of Case 2 contained almost 400 times as much neutral fat as those in Case 1. From these data, it appears that there is a marked lack of fatty material in the body of the child with generalized lipodystrophy, which confirms the clinical and pathological (gross and microscopic) diagnosis. Further, we may conclude that this deficiency apparently is specifically due to a lack of neutral fat fatty acids from the various tissues studied. The name "lipohistiodiarsis" (lack of fat in the tissues) has been suggested to describe this phase of the condition.

11545

Increased Serum Phosphatase Activity Without Hyperbilirubinemia after Ligation of Hepatic Ducts in Dogs.

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In man with complete obstruction of the common bile duct, hyperbilirubinemia is associated with markedly increased phosphatase activity of the serum. When obstruction of the common duct is incomplete (as frequently in choledocholithiasis, cholangitis) or in intrahepatic biliary tract obstruction (hepatic metastases, etc.), little or no jaundice may result but the serum phosphatase is usually

* Supported in part by a grant from the Josiah Macy, Jr., Foundation.

distinctly elevated. In hepatitis ("catarrhal" jaundice), even marked hyperbilirubinemia is associated quite regularly with comparatively little rise in serum phosphatase activity.¹

Corresponding changes in serum phosphatase have been produced experimentally in the dog: Ligation with complete obstruction of the common bile duct^{2,3,4} results in extremely high serum phosphatase levels; hepatitis produced by hepatotoxic drugs^{4,5,6} or by *Leptospiral* inoculation⁴ causes increases in serum phosphatase generally considerably less than those observed after common duct ligation, though hyperbilirubinemia may be as marked or more marked.[†]

The dissociation of serum phosphatase and serum bilirubin levels observed clinically with incomplete obstruction of the biliary tract has been little studied experimentally but was reproduced by Freeman, Chen and Ivy⁴ in 2 dogs following ligation of hepatic ducts draining approximately $\frac{1}{3}$ of the liver. Though jaundice did not develop, the serum phosphatase rose to 25.8 and 37.9 Bodansky units per 100 cc, respectively; in the one instance followed, there was a spontaneous, gradual fall to almost normal levels after 2 months. Confirmatory data are recorded here together with such additional studies on the urine as bear upon the interpretation of the blood changes.

Methods. In 4 dogs following nembutal anesthesia, the hepatic ducts were identified by aspiration of bile and a variable number (draining $\frac{1}{5}$, $\frac{1}{4}$ or $\frac{1}{2}$ of the liver) were tied off with silk. Periodic postoperative examinations were made of the serum for phosphatase (Bodansky method), total cholesterol (Bloor method) and bilirubin, and of the urine for "alkaline" phosphatase (King

¹ For references and additional data see Gutman, A. B., Olson, K. B., Gutman, E. B., and Flood, C. A., *J. Clin. Invest.*, 1940, **19**, 129.

² Bodansky, A., and Jaffe, H. L., *Proc. Soc. Exp. Biol. and Med.*, 1934, **31**, 1179.

³ Armstrong, A. R., King, E. J., and Harris, R. I., *Canad. M. A. J.*, 1934, **31**, 14.

⁴ Freeman, S., Chen, Y. P., and Ivy, A. C., *J. Biol. Chem.*, 1938, **124**, 79.

⁵ Armstrong, A. R., and King, E. J., *Canad. M. A. J.*, 1935, **32**, 379.

⁶ Bodansky, A., *Enzymolog.*, 1937, **3**, 258.

[†] An interesting exception is *m*-toluylenediamine jaundice in dogs, which is associated with very high serum phosphatase and cholesterol values.⁶ This type of jaundice, however, appears to be partly hemolytic, partly due to stasis of bile in the finer biliary radicles rather than to parenchymal injury.⁷ A somewhat analogous condition, with high serum phosphatase, is seen clinically in certain drug-hypersensitive cases of post-arsphenamine jaundice, in which liver biopsies show obstruction of the intrahepatic biliary tract.⁸

⁷ Naunyn, B., *Mitt. Grenzgeb. Med. u. Chir.*, 1919, **31**, 537.

⁸ Hanger, F. M., and Gutman, A. B., *J. A. M. A.*, in press.

and Armstrong method) and bile. The animals were sacrificed after 4-9 weeks and the presence of intact ligatures with dilatation of the proximal hepatic ducts verified at postmortem.

Control preoperative serum and urine analyses were obtained. Nembutal anesthesia was found not to affect serum phosphatase values. Urine samples were obtained by catheter to avoid contamination with phosphatase- and bile-rich feces.

Results. The serum phosphatase activity increased within 24 hours after operation to reach 5-20-fold levels in 4 days and a peak usually between the first and second week; the maximum level varying with the number of hepatic ducts ligated. A spontaneous decline followed, with return to approximately normal levels after some 2 months. A roughly parallel rise in the total cholesterol content of the serum was noted though the return to normal was more rapid. No significant increase in serum bilirubin occurred except a transient rise to 0.5 mg % on the fourth day in Dogs 1 and 3. Bile pigments appeared in the urine within 24 hours after operation and persisted for many weeks, gradually decreasing in amount. Significant "alkaline" phosphatase activity was not present in any urine specimens with the exception of small amounts in the urine of Dog 3. At postmortem, the liver lobes tied off appeared to be normal grossly and showed only slight changes histologically.

Discussion. It is not clear whether the dissociation of serum phosphatase and bilirubin levels observed clinically with incomplete biliary obstruction and reproduced in these experiments should be regarded as incompatible with the "phosphatase retention" theory—that the increase in serum phosphatase levels in obstructive jaundice is due to retention of phosphatase normally excreted in the bile. One factor that might cause such dissociation in blood levels following

TABLE I.
Phosphatase Activity (P, in Bodansky units/100 cc) and Total Cholesterol (C, in mg/100 cc) of the Serum after Ligation of Hepatic Ducts in the Dog.

Time post-op.	Dog 1 ¼ liver ligated		Dog 2 ½ liver ligated		Dog 3 ½ liver ligated		Dog 4 ¼ liver ligated	
	P	C	P	C	P	C	P	C
Pre-op.	3.7	120	4.1	154	3.4	138	2.8	176
1 day	7.3	172						
4 "	26.2	171	19.6	182	61.7	250		
1 wk			21.7	247			29.4	235
1½ "	16.9	192	22.1		74.0	435		
2½ "	11.2	117	9.9	154	49.1	268	25.1	207
4 "	6.8		6.8	149	28.2		13.4	205
5 "	4.4				14.7	195	7.8	169
7 "					9.2	149	7.2	
9 "					4.5		3.7	138

bile retention would seem to be the differential excretion of bilirubin and phosphatase in the urine.

In *man* with incomplete obstruction of the biliary tract and therefore only moderate retention of bile, urinary excretion of bile pigments may increase sufficiently to maintain the patient virtually free of jaundice. The human kidney is impermeable to serum phosphatase, however, and this difference in available excretory channels appears to be partly responsible for the observed dissociation of serum phosphatase and bilirubin levels. In the *dog*, the blood and urine studies recorded here suggest that the same factors are responsible for the analogous dissociation in serum levels. By varying the number of hepatic ducts tied off, a positive correlation between the serum phosphatase level and the degree of obstruction could be demonstrated. The serum phosphatase values in our dogs are relatively high as compared with man but this corresponds with the extraordinarily high values in dogs with complete biliary obstruction; the serum bilirubin levels are relatively low, due to the greater clearance of the dog kidney. In the *cat*, on the other hand, "alkaline" phosphatase as well as bilirubin appears in the urine⁹ and complete obstruction of the common bile duct results in comparatively little rise in either serum phosphatase or bilirubin.¹⁰

Factors other than differential renal excretion may contribute to the dissociation of serum phosphatase and bilirubin levels following bile retention since their level in the serum represents a quite complex dynamic equilibrium between the rate of secretion into the blood stream and the rate of excretion (by one or more channels), metabolism or deposition in the tissues. Though not open to direct measurement, the important influence of these latter factors on the serum phosphatase level is indicated by the wide range in serum phosphatase values in man with complete common duct obstruction.¹¹

We conclude that the occurrence of increased serum phosphatase activity with little or no jaundice following incomplete biliary tract

⁹ Flood, C. A., Gutman, E. B., and Gutman, A. B., *Am. J. Physiol.*, 1937, **120**, 696.

¹⁰ Cantarow, A., Stewart, H. L., and McCool, S. G., *PROC. SOC. EXP. BIOL. AND MED.*, 1936, **35**, 87.

[†] We are concerned here only with incomplete biliary obstruction, not with liver parenchymal injury as a cause of dissociated blood values. Bodansky has shown^{6,11} that in the latter case there is selective impairment of different liver functions by various hepatotoxic drugs.

¹¹ Bodansky, A., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **42**, 800.

obstruction is not inconsistent with the "phosphatase retention" theory. As to whether the increased serum phosphatase is of hepatic or osseous origin, our results do not exclude the one or the other possibility since occlusion of the excretory biliary channels would tend to cause retention of bile constituents of both hepatic and extra-hepatic origin.

Summary. Ligation of hepatic ducts in 4 dogs resulted in increased phosphatase but not bilirubin in the serum, increased bilirubin but not phosphatase in the urine. The dissociation in the blood is thought to be due largely to differential renal excretion and to be consistent with the "phosphatase retention" theory.

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**Breast Cancer Produced in Male Mice of the C57 (Black)
Strain of Little.**

GRAY H. TWOMBLY. (Introduced by C. P. Rhoads.)

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Bittner¹ has shown that hybrids from a cross between a mouse from a strain having a high incidence of mammary cancer and one from a low tumor strain have a high or low incidence of breast cancer depending on what type of mother they nurse. If the nursing mother comes from the strain having the high incidence of spontaneous tumors, a large percentage of the female hybrids will develop mammary cancer. If they are nursed, on the other hand, by a female from the low tumor strain, very few will develop breast cancer. Female mice from a high cancer strain nursed by their own mothers have a high incidence of mammary cancer while if they are foster-nursed by a mouse from a low tumor strain the chance that they will develop breast cancer will be materially reduced.

An attempt was made to confirm this observation on a different strain of animals. Mice of the RIII (Paris) strain of Dobrovolskaia Zavadskaia were given to a female of the C57 (black) strain of Little to nurse while the young of the latter were given to the RIII mother. The RIII females have an incidence of spontaneous mammary cancer of 70% in virgin females. The incidence in C57 black females is less than 1%. The incidence of breast

¹ Bittner, J. J., *Am. J. Ca.*, 1939, **35**, 90.

cancer occurring in these 2 groups of animals appears to confirm Bittner's observations but the number of animals is as yet not very statistically significant and consequently will be reported later in another place.

Since litters were shifted within less than 12 hours of birth when the sex was often hard to determine, many males which had been foster-nursed by mothers of the other strain became available. It has been proved² that male mice of a strain showing a high incidence of spontaneous breast cancer in the females will develop cancer more quickly than their littermate sisters if a crystal of oestrone weighing 0.1 mg is implanted in them subcutaneously on the tenth day after birth. Because this procedure was easy and the mice were available the C57 males nursed by RIII females were so treated.

Many animals have been used, a fair proportion of which have died of hyperestrinism before they were old enough to show tumors. Some are still too young for tumors to have developed. Nine males so treated have had spontaneous mammary cancer, 8 of which have been proved histologically. The ninth died in the night and was eaten by his cage-mates before morning. The earliest tumor appeared at 7½ months after birth in a mouse implanted with 0.18 mg of crystalline oestrone. The oldest mouse to develop a tumor did so 11 months after birth after a dose of 0.09 mg of oestrone. The average age at which tumors appeared was 9 months. So far 27 animals have lived 9 months or longer or have developed tumors before the ninth month. Fourteen of these animals are still alive. In this small series then we may say that by foster nursing C57 black male mice with RIII (Paris) mothers and implanting them at 10 days of age with a crystal of oestrone weighing 0.07 to 0.18 mg, 9 out of 27, or 33%, have developed breast cancer.

This observation assumes greater importance when one considers that mammary cancer has not been observed previously in C57 black male mice. Haagensen in a personal communication reports the treatment of 107 males with maximal doses of oestrone benzoate in oil twice a week from 10 days of age to death without producing a single tumor. Gardner has had 3 tumors in 250 mice treated. It would seem that breast cancer in the animals reported in the present communication is due not only to hormonal stimulation of the male breast tissue but to some agent or influence transmitted in the mother's milk other than oestrogenic substances.

² Twombly, G. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **40**, 430.

Production of Gastric and Duodenal Ulcers in the Cat by
Intramuscular Implantation of Histamine.*

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The finding of Code and Varco¹ that prolonged stimulation of gastric secretion could be obtained by the injection of a histamine beeswax mixture into dogs offered a means of testing whether endogenous gastric secretion could produce ulcers of the stomach or duodenum. Cats were chosen for this investigation, because it had been found in a recent study in this laboratory that these animals developed erosions and ulcerations of the stomach and duodenum quite readily when 0.4% hydrochloric acid was instilled daily into the stomach through a fistula (Walpole). It seemed possible that a profitable comparison might be made between ulcer production in response to endogenous and exogenous acid.

Experimental Procedure. The problem was approached by first studying the effects of single injections of the histamine beeswax mixture on gastric secretion and then observing the effects produced by chronic histamine administration. The effect of the histamine beeswax mixture upon gastric secretion was studied in 3 cats with a standard type of gastrostomy.

Controls. Control observations were made with 2 of these animals some days prior to the injections of histamine. The continuous secretion of the fasting stomach was collected every 8 hours for 24 hours. Several days later each cat was given an intramuscular injection of plain beeswax mixture equivalent in amount to that in a dose of the histamine beeswax mixture and the continuous gastric secretion was collected every 8 hours for 24 hours. In addition, in 3 control cats beeswax alone was implanted daily intramuscularly over periods varying from 8 to 23 days. In each instance, the cat gained weight and its general health appeared

* This research was supported by grants from the Committee on Scientific Research of the American Medical Association (grants 526 and 556) and by a grant from the Graduate School of the University of Minnesota; also by a grant for technical assistance by the Work Projects Administration, Official Project No. 665-71-3-69, Sub-project 258.

¹ Code, C. F., and Varco, R. L., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 475.

satisfactory. One of these cats was killed and subjected to a careful necropsy. There was no ulcer. The other cats continued in good health. During the period of this study, 13 cats kept in the laboratory for other purposes have been subjected to necropsy. No ulcers were found. The cat has been used periodically as an experimental animal in the surgical laboratory over a period of years. No spontaneous ulcers have been observed.

Method. The acidity of the gastric juice was determined by colorimetric titration. In both groups the quantity of secretion obtained and the maximum free acidity produced were considerably lower than that following stimulation with histamine in beeswax. The maximum free acidity of the gastric juice following administration of plain beeswax was 48 clinical units, and of 6 samples collected 3 had no free acid. The maximum free acidity attained by the fasting stomachs was 66 clinical units, and of 6 samples collected 3 contained no free acid. The experiments indicate that plain beeswax in the dose given had no effect upon gastric secretion.

As a routine, the dose of histamine used in the beeswax mixture was 20 mg of the free base. This was injected in divided portions into the muscles of the back. In the cats with gastric fistulae, following administration of this dose, either fractional samples were collected or the entire continuous secretion was taken at hourly intervals for 24 hours. Food was withheld for 24 hours preceding injection. As in the dog (Code and Varco) there was a constant copious secretion of gastric juice. After a short lag during the first hour, the free acid rose to values of more than 100 clinical units but usually fell later to a somewhat lower range. In only 2 samples of 108 collected was free acid absent.

To determine the effect of this prolonged abundant flow of gastric juice with high free acidity upon the stomach and duodenum, 7 healthy cats weighing 4 to 8 lb were given each a daily dose of 20 mg of histamine in beeswax injected intramuscularly. The animals were fed each morning and the injections made several hours later. Adequate fluids were supplied by subcutaneous administration of normal saline solution. The animals were sacrificed when they appeared obviously ill, or refused food on 2 successive days, or after blood was noted in several specimens of vomitus.

Histamine in beeswax frequently produced no obvious reaction, but occasionally the injection was followed immediately by a typical chain of symptoms. This consisted of restlessness, increased respirations, and vomiting associated often with salivation and passage of a loose stool. The occurrence of these symptoms seemed to

depend more upon the batch of histamine mixture used than upon the individual animals. Recently, with increased experience in preparation of the material, reactions have been less frequent. In all animals, however, vomiting occurred some time during the course of injections and when not associated with the immediate reaction took place several hours after the injection. The vomitus usually contained free acid.

These 7 cats receiving the histamine beeswax mixture were sacrificed at periods ranging from 3 to 25 days after beginning the injections, the total amount of histamine given ranging from 60 to 480 mg. At necropsy there were erosions or acute ulcers of the stomach or duodenum, or both, in all animals. Two cats had lesions of the duodenum only, 2 had lesions of the stomach only, and 3 had lesions of both stomach and duodenum. In 3 animals there were perforated ulcers, two in the duodenum and one in the stomach. Gastric lesions were limited to the antrum. It seemed obvious that the animals had been sacrificed at various stages of ulcer formation.

Comment. Histamine in beeswax prepared according to the method of Code stimulated a sustained copious flow of gastric juice containing free acid when injected intramuscularly into cats. Repeated injections of histamine in saline solution have been reported as failing to produce ulceration in the gastro-intestinal tract in the dog² and also in the cat.³ Repeated single daily doses of histamine in beeswax in this study were effective in the cat in producing erosions and all stages of ulceration including acute perforation. In one dog tested, ulceration occurred in the duodenum.† These findings suggest the importance of the gradual liberation of histamine from beeswax in maintaining a constant and fairly uniform stimulation of gastric secretion, as opposed to the intermittent stimulation afforded by periodic injections of histamine in watery solution.

² Orndorff, J. R., Bergh, George S., and Ivy, A. C., *Surg. Gynec. Obstet.*, 1935, **61**, 162.

³ Heinlein, H., and Kastrup, H., *Z. f. d. ges. exp. Med.*, 1938, **102**, 517.

† Since this paper was written intramuscular implantation of histamine has been done in an additional cat and in another dog. Both animals were killed and autopsied when it was apparent that they were ill. The cat had a large ulcer in the fundus 12 days after the administration of histamine was begun. The dog had a small ulcer .5 cm in diameter in the first portion of the duodenum 4 days after histamine administration was begun.

Relationship Between Insulin Dosage, Duration and Degree of Hypoglycemia and Production of Brain Damage.*†

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This report deals with the relation of insulin dosage, duration and degree of the ensuing hypoglycemia, and the resulting brain damage produced. Observations of a paradoxical reaction with insulin are also recorded.

It is well established from the work of Scott and Dotti,¹ Zucker and Berg,² and many others, that from 20 to 60 minutes after the injection of insulin, the blood sugar reaches its lowest level and remains there with minor fluctuations for a period depending upon the amount of insulin given. With insulin doses such as we used, of from 10 to 20 units per kilo of bodyweight, the hypoglycemia persists from 10 to more than 24 hours. However, independent of the hypoglycemia certain clinical symptoms occur which indicate a progressive loss of function, in a phylogenetic order, from the higher cortical areas to the lower or medullary centers.³⁻⁶ In this connection, Frostig⁴ described 4 stages of hypoglycemia in man after large doses of insulin, based on impairment of the function of (1) cerebral cortex, (2) basal ganglia and thalamus, (3) mid-brain, and (4) medulla. In our previous report,⁷ certain interesting data became apparent bearing on the problem of insulin dosage in relation to brain damage.

The method used has been described in a previous report.⁷ Briefly stated, doses of insulin were given subcutaneously to cats fasted 18 hours and not previously treated with insulin. Complete

* Insulin was kindly furnished by Eli Lilly & Co.

† We are indebted to Dr. C. H. Thienes of the Department of Pharmacology for the use of laboratory space and many helpful suggestions.

¹ Scott, E. L., and Dotti, L. B., *Arch. Int. Med.*, 1932, **50**, 511.

² Zucker, T. F., and Berg, B. N., *Am. J. Physiol.*, 1937, **119**, 531.

³ Angyal, L. V., *Z. Neur. and Psychiat.*, 1937, **157**, 35.

⁴ Frostig, J. P., *Arch. Neur. and Psychiat.*, 1938, **39**, 219.

⁵ Himwich, H. E., Frostig, J. P., Fazekas, J. F., and Hadidian, Z., *Am. J. Psychiat.*, 1939, **96**, 371.

⁶ Ziskind, E., and Tyler, D. B., in preparation.

⁷ Ziskind, E., and Tyler, D. B., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **43**, 734.

quarter-hour notations of the neurological state of the animal were made. When the animal showed signs of critical medullary decompensation, small amounts of glucose (100-200 mg) were given intraperitoneally as needed, in order to restore the circulation, pulse and respiration, but still maintain a severe hypoglycemia. The hypoglycemia was terminated after 9 to 20 hours with glucose. We considered those animals had brain damage which showed the irreversible clinical symptoms of "decortication" and "decerebration" described elsewhere.⁷ In this report when we describe a "stage" of hypoglycemia such as the "medullary stage," we refer to the neurological symptoms at that time.

1. *The Relation Between Insulin Dose and Incidence of Brain Damage.* Column III of Table I shows that the greater the dosage the greater the incidence of residual brain damage. In animals receiving 10, 15 and 20 units of insulin per kilo, the incidence of brain damage was respectively 30%, 33%, and 62%. These results cannot be correlated with the duration of hypoglycemia or the period of coma (columns IV and V). These findings correspond to those previously reported by Yannet.⁸

TABLE I.

I	II	III	IV	V
Insulin dose	Time before myoclonic jerks	Incidence of residual brain damage	Hr of hypoglycemia	Hr coma
20 u./kg	4.16 (20)	62% (13)	11	7
15 u./kg	3.67 (43)	33% (33)	13	9
10 u./kg	3.28 (20)	30% (20)	7.5	4.5
Less than 5 u./kg	2.24 (12)	—	—	—

Number in parenthesis indicates number of animals.

2. *The Relation of Duration of Medullary Stage to Brain Damage.* Residual brain damage did not occur in our series irrespective of the dose of insulin unless the animal was in the "medullary stage" for not less than 100 minutes. This medullary stage (Stage IV of Frostig) is characterized by pin point pupils, bradycardia, respiratory irregularities especially Cheynes-Stokes respiration, flaccidity, and finally symptoms of circulatory collapse. However, the time that the animal must be kept in this stage to produce brain damage varies indirectly with the body temperature of the animal. These findings do not lend support to the thesis that insulin *per se* is toxic to the brain cell.

3. *Paradoxical Relation of Increasing Dose of Insulin and Disappearance of Cerebral Functions.* The larger the dose of insulin

⁸ Yannet, H., *Arch. Neur. and Psychiat.*, 1939, **42**, 395

(within the limits of our experiments) the longer the latent period before cortical functions disappear, as gauged by the loss of consciousness and the time of appearance of the first myoclonic jerks. (Column II of table.) The average time of appearance of the first myoclonic jerks in cats receiving 5 u/kg or less was 2.24 hours, 10 u/kg 3.28 hours, 15 u/kg 3.67 hours, and 20 u/kg 4.16 hours. We are unable to suggest the reason for this reaction.

Conclusions. 1. The larger the dose of insulin the greater the incidence of brain damage. However, irrespective of the insulin dose, brain damage did not occur in our animals unless they were kept in the "medullary stage" of hypoglycemia for at least 100 minutes. 2. In cats, not previously treated with insulin, the larger the dose the longer time it took for the appearance of neurologic signs of hypoglycemia.

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Elimination of Metrazol.

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Pharmacological and clinical evidence points to the fact that metrazol is very rapidly detoxicated in the body. The intravenous administration of a convulsive dose produces typical clonic convulsions from which the animal rapidly recovers.

There is a possibility that the metrazol might be excreted by the kidneys, and so our first step consisted in eliminating this possibility. Chemical analysis of the urine of cats receiving convulsive doses of metrazol showed none of the drug to be present in the urine. Bilaterally nephrectomized cats showed the same reaction to a convulsive dose of metrazol as they did before the performance of the nephrectomy. Hinsberg has shown that practically no metrazol is excreted by the intestinal route. It therefore seems logical that metrazol is not excreted but is detoxified.

The liver has generally been assumed to be the locale for drug detoxication. We therefore administered phosphorus to cats. Cats treated in this way died from the administration of a dose of metrazol which formerly produced only slight convulsions. The role of the liver was further tested by a comparison of the dose required to

produce convulsions when the drug was infused into the marginal ear vein or the portal vein of rabbits. In all cases a larger amount was required to produce convulsions when administered by the portal route. This evidence seems to establish the fact that metrazol is detoxified rather than excreted and that the liver plays an important rôle in the detoxication process.

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Coronary Occlusion. II. Efficacy of Papaverine Hydrochloride in Treatment of Experimental Cardiac Infarction.

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Papaverine has been suggested repeatedly as a drug of therapeutic value in the treatment of coronary artery disease.¹⁻⁴ This recommendation has been based on the thesis that the vaso-dilator action of papaverine would increase local blood flow. The anginal attack is thus relieved, or the ultimate size of the myocardial scar is minimized as a result of the improved nutrition.

Allen and MacLean⁴ stated that the pain in peripheral arterial embolization is due not only to the presence of the embolus, but also to the attendant widespread reflex vascular spasm. The extensive ischemia that they observed was more marked than could be accounted for by the occlusion of the main vessel alone. In arterial embolization papaverine is said to have effects analogous to sympathectomy, *i.e.*—relaxation of the vaso-spasm. The use of papaverine has been suggested also in cerebral, pulmonary and mesenteric occlusion.^{3,4} Mulinos, Shulman and Mufson⁵ found that vaso-spasm of Reynaud's disease was relieved by large doses of papaverine hydrochloride intravenously, doses which did not lower the blood pressure but which resulted in a moderate acceleration of the heart rate.⁶ Gruber and Robinson⁷ noted that papaverine in small

¹ Semler, R., *Med. Welt.*, 1928, **2**, 335.

² Neu, J., *Therap. d. Gegenw.*, 1927, **68**, 564.

³ De Takats, G., *J. A. M. A.*, 1936, **106**, 1003.

⁴ Allen, E. V., and MacLean, A. R., *Proc. Staff Meet. Mayo Clinic*, 1935, **10**, 216.

⁵ Mulinos, M. G., Shulman, I., and Mufson, I., *Am. J. Med. Sci.*, 1939, **197**, 793.

⁶ Mulinos, M. G., and Shulman, I., *J. Pharm. Exp. Therap.*, 1939, **63**, 27.

⁷ Gruber, C. M., and Robinson, P. I., *J. Pharm. Exp. Therap.*, 1929, **37**, 429.

doses caused an increase in the height of the contractions in the isolated perfused terrapin heart. Macht⁸ observed that small doses caused slowed, more powerful contractions of the isolated frog's heart.

Coronary occlusion, whether due to embolus, spasm, or ligation, results in a central area of almost complete ischemia which is surrounded by a halo of myocardial edema and reflexly spastic vessels. These peri-infarctial coronary vessels are of importance because they supply blood to the adjacent myocardium and also because they are potential anastomotic connections with the infarcted area (Wearn⁹). It is to be expected, therefore, that vasodilators which decrease the vaso-spasm should prove beneficial by increasing the anastomotic circulation, and also by lessening the peri-infarctial edema, thus diminishing the ultimate size of the infarct. No experimental work has been done in support of the contention that papaverine exerts such a beneficial effect on the course of occlusive coronary disease. The present study attempts to determine the effect of papaverine upon the size of an experimentally produced myocardial infarct. Gold, Travell and Modell¹⁰ and Fowler, Hurevitz and Smith¹¹ have made similar studies employing aminophylline as the vasodilator.

Method. Using the method described previously,¹² the left branch of the left anterior descending coronary artery was ligated aseptically in 22 cats under sodium pentobarbital anesthesia. An attempt was made to tie the vessel at the same point in each animal. Standard 3 lead electrocardiograms, white and differential blood counts, and determinations of the sedimentation rate were made pre-operatively, and at intervals in the post-operative life of the cats. Following the ligation alternate cats received 5 mg per kilo of papaverine hydrochloride (1 cc = 30 mg)* intramuscularly twice a day for a period of 2 weeks (Sundays excepted). The interval cats were kept as controls and were treated analogously except for the injections. All the cats remained in good condition until they were sacrificed.

⁸ Macht, D. I., *Arch. Int. Med.*, 1916, **17**, 786.

⁹ Wearn, J. T., Harvey Lecture, 1940.

¹⁰ Gold, H., Travell, J., and Modell, W., *Am. Ht. J.*, 1937, **14**, 248.

¹¹ Fowler, W. M., Hurevitz, H. M., and Smith, F. M., *Arch. Int. Med.*, 1935, **56**, 1242.

¹² Scott, W., Leslie, A., and Mulinos, M. G., *Am. Ht. J.*, 1940, **19**, 719.

* Generously contributed by Eli Lilly and Company.

TABLE I.
Infarct Size and Its Relation to Age of Infarct and Administration of Papaverine.

Treated cats			Untreated cats		
Cat No.	Days	Infarct area, cm ²	Cat No.	Days	Infarct area, cm ²
18	7	0.88	9	24	1.33
29	7	3.19	5	27	1.59
25	14	3.76	3	28	1.62
16	15	2.24	12	29	1.22
13	20	1.76	27	30	0.55
26	20	1.52	10	30	2.06
6	21	2.91	11	30	1.30
14	21	0.80	7	33	1.07
4	29	3.64	8	44	2.15
19	101	0.39	9	47	1.58
			10	50	4.40
			21	56	None seen
10	Avg 25.5	Avg 2.11 $\sigma = 0.363$	12	Avg 35.7	Avg 1.57 $\sigma = 0.324$

From 6 to 102 days after operation, the cats were killed by the intravenous injection of chloroform. The hearts were excised, the infarct delineated with ink and a contact tracing of each infarct was made. The areas of the tracings, shown in Table I, were determined by means of a planimeter. There is no obvious correlation between the size of the infarct and its age.

Results. The treated cats experienced some immediate pain from the injection of the papaverine hydrochloride, which has a pH of 2, but showed no other untoward effects. There was no depression, vomiting, or loss of appetite.

As shown in the Table, the area of infarct averaged 34% larger in the papaverine treated cats than in the uninjected controls. This difference falls well within the average deviation of either series and is consequently considered as accidental. In large part the size of the infarct depends in the different cats upon the anatomical distribution of the coronary vessel which is ligated at the operation. The differences in the area of the infarcts are shown in the Table as a scatter of from 0.39 sq cm to 3.76 sq cm for the papaverine treated cats and of from zero to 4.40 sq cm for the control animals. A larger series might be desirable. However, more data would merely result in lowering of the average deviation of infarct size, without throwing additional light upon the effect of the drug. Our figures indicate that infarct size cannot be used as a criterion of coronary vasodilator action and therefore it is felt that the coronary vasotropic effect of papaverine must be studied by other methods as well. Papaverine is being compared by us with nitroglycerine

and theophylline for its effects upon the electrocardiogram with and without induced anoxemia. The length of time necessary for the recovery of the electrocardiogram and for the return of the white blood cell count and sedimentation time to normal was roughly proportional to the size of the infarct, and independent of the administration of the papaverine. From our failure to demonstrate any reduction in the size of the infarct after coronary ligation, it cannot be concluded that papaverine hydrochloride has no place in the clinical treatment of coronary occlusion or of angina pectoris.

Conclusion. The daily injection of papaverine hydrochloride (5 mg per kilo) into cats for 2 weeks did not alter significantly the size of the infarct resulting from the ligation of the left branch of the left anterior descending coronary artery.

It is suggested that because of the greatly variable amount of cardiac tissue involved in each ligation this method of study is too crude to detect any "clinical" improvement that the drug may have exerted.

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Anaphylactic Shock and Susceptibility to Histamine Poisoning in the Cotton Rat *Sigmodon hispidus littoralis*.*

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The Eastern cotton rat *Sigmodon hispidus hispidus* and the Florida cotton rat *Sigmodon hispidus littoralis* have come into prominence as laboratory animals because of their reported susceptibility to the virus of poliomyelitis.^{1 2} The cotton rat is a small rodent and apparently it is capable of adaptation to laboratory life. It is therefore of interest to explore its usefulness for other experimental purposes. Reports on a natural trypanosome infection of the Florida cotton rat,³ on the susceptibility of this animal to diph-

* This work was supported by a grant from the Philip Hanson Hiss, Jr., Memorial Fund.

¹ Armstrong, C., *Pub. Health Rep.*, 1939, **34**, 1719.

² Jungeblut, C. W., and Sanders, M., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **44**, 375.

³ Culbertson, J. T., *J. Parasit.*, in press.

theric toxin,⁴ and to infection with the tubercle bacillus⁵ have been made. The present communication describes attempts to produce anaphylactic shock in the Florida cotton rat. In addition to testing for hypersensitivity, the serums of some of the animals, obtained after sensitization, were tested for precipitin content. The animals subsequently were subjected to intravenous injections of histamine.

Sheep serum as an anaphylactogen: One or 2 sensitizing injections of sheep serum were given intravenously to 8 cotton rats. The total amount used for sensitization ranged from 0.05 to 0.6 cc. After an incubation period, varying from 17 to 29 days, the animals were reinjected intravenously with 0.25 to 0.50 cc of sheep serum. In no case was any reaction obtained following the shocking injection of antigen. Six of these animals were retested for sensitivity 17 days later by the intravenous injection of 0.3 to 0.5 cc of sheep serum. The animals were again equally refractory to shock.

Five of the animals were bled 19 or 25 days after the last shocking injection of antigen, and their serums tested for precipitins to sheep serum. In 3 of the serums no precipitins could be demonstrated; in 1 serum there was a trace with antigen diluted 1:20, while the last serum reacted to give a definite precipitate with antigen diluted 1:10, 1:40, and 1:160 and a trace with antigen diluted 1:640.

Whole egg white as an anaphylactogen: Two sensitizing injections of 0.5 cc of 25% whole egg white were given 3 days apart to 9 cotton rats. The first injection was given intravenously, the second intraperitoneally. Five of the 9 animals had previously been used in the sheep serum experiments. After a 21-day incubation period, all 9 animals received 0.5 cc of the same egg white solution intravenously. Seven of the cotton rats failed to show any reaction. One animal was listless for about 20 minutes, sitting in one corner of the cage and responding only sluggishly to prodding. The last animal showed a greatly increased rate of respiration for about 5 minutes after injection. It then appeared normal for about 15 minutes when it was observed that the animal had become limp and could not be aroused to activity. Respirations were slow but not labored. The animal lay on its side, became progressively weaker with slower respiration during another 15 minutes and then stopped breathing, 35 minutes after the injection of egg white. Autopsy revealed no gross lesions in the heart or lungs. Each pleural cavity contained a drop of blood or bloody fluid, and there was a small amount of serosanguinous fluid in the peritoneal cavity. The

⁴ Jungeblut, C. W., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **43**, 479.

⁵ Steinbach, M. M., and Duca, C. J., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **44**, 288.

mucosa of the stomach had one area of hemorrhage 4 mm in diameter and one of the Peyer's patches of the small intestine was studded with petechial hemorrhages. The type of death and the paucity of findings at autopsy suggested the picture of anaphylaxis in the rat.

Four of the 9 animals sensitized to egg white were bled from the heart 4 hours before the intravenous shocking injection of antigen. Serums from these bloods were tested for precipitins. Three of the serums contained no demonstrable precipitins, while the fourth showed only a trace of precipitate when tested with antigen diluted 1:20 and 1:100.

Histamine shock: Eight cotton rats were tested for their susceptibility to histamine (ergamine acid phosphate, Burroughs Wellcome & Co.). The drug was dissolved in 0.85% NaCl so that each cubic centimeter contained 1 mg. The cotton rats, weighing between 130 and 175 g, were injected intravenously with 0.4 to 1.5 mg. The minimal lethal dose proved to be roughly 1 mg of histamine for a 130 g cotton rat, or approximately 0.8 mg per 100 g. This amount killed 4 out of 5 animals, whereas 0.6 mg per 100 g respiratory rate in the 3 animals tested. There was considerable of cotton rat failed to produce more than transitory increase in individual variation in the mode of death. In 1 animal (weight 132 g) death occurred in 3 minutes following injection of 1 mg of histamine. It was characterized by frothing at the nose and labored respiration and by tonic and clonic convulsions and opisthotonos. At autopsy the lungs were slightly distended and hemorrhagic. A second animal (weight 175 g) died one hour following the intravenous injection of 1.5 mg. This animal did not show discharge of froth from the nose and the respirations, although rapid, were not labored. It lay on its side, prostrated, for 45 minutes before death. On autopsy the lungs appeared normal. There were many petechial hemorrhages in the Peyer's patches and also scattered through the small intestine. A third rat, also 130 g in weight, died in 3 hours following the injection of 1 mg. In this case, difficulty of respiration was also absent. Periods of complete prostration alternated with periods of violent tonic and clonic convulsions. The only findings at autopsy were petechial hemorrhages in Peyer's patches. A fourth cotton rat, which had also been given 1 mg of histamine, showed only some increase in the rate of respiration for 30 minutes following injection, but looked sick and listless the following day and died 24 hours later without showing any lesions at autopsy.

The potency of the histamine was tested in 3 guinea pigs weighing 700 g. Two of these animals died acutely following the injection of 0.4 mg while the third survived 0.3 mg.

Conclusions: The cotton rat was found relatively refractory to anaphylactic shock. In this respect it resembles the ordinary laboratory rat. Low titered precipitins occurred in 3 of the 9 serums tested after sensitization. The minimal lethal dose of histamine intravenously was approximately 0.8 mg per 100 g for the cotton rat. This is 15 times the quantity required to kill a guinea pig, but is 100 times less than that which has been reported lethal for the rat.

11552

Relation Between Volume of Vehicle and Chick Comb Response to Androsterone.*

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From the Laboratories of the Mount Sinai Hospital, New York.

It was suggested previously (Frank, Klempner and Hollander¹) that, in the use of sesame oil as a vehicle in our bioassay method for androgens, a reduction in the volume of vehicle from 0.1 cc to 0.05 cc was one of the factors which contributed to the improvement in response. Subsequently, the possibility presented itself that a further reduction in this volume might effect further improvement, as manifested by increased comb growth for a given dose of androgen. Accordingly, we have investigated the response elicited by the application of various dosages of androsterone in 0.05 cc and in 0.02 cc† of oil, applied daily, in paired experiments run simultaneously. In all other respects, the experimental conditions were exactly the same as in our last report (*loc. cit.*).

The results of such paired experiments are summarized in Table

* This investigation was supported in part by a grant from the Friedsam Foundation.

¹ Frank, R. T., Klempner, E., and Hollander, F., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, **38**, 853.

† In order to facilitate the application of these small volumes, as well as to control accuracy of delivery, a simple mechanical device was attached to the syringes used. This device, constructed by Mr. Vondrak, chief technician of laboratories, Mount Sinai Hospital, will be described elsewhere.

TABLE I.
Effect of Further Reduction in Volume of Vehicle (Sesame Oil) in the Bioassay
of Androgens by the Chick Comb Method.

Series No. (1)	Androgen dosage (γ) (2)	Vol. of oil (cc) (3)	No. of chicks (4)	Mean comb wt		Response to androgen application (comb wt corrected for controls)		Improvement in response (IR) ² (9)
				Treated chicks W (mg) (5)	Control chicks W _c (mg) (6)	(W-W _c) (7)	% of W _c ¹ (8)	
27	10	.05	25	34.0	18.5	15.5	84	+52
		.02	25	43.6	(28)*	25.1	136	
28	10	.05	30	31.1	17.5	13.6	78	+63
		.02	30	42.2	(26)	24.7	141	
29	10	.05	30	31.0	19.3	11.7	61	+89
		.02	30	48.3	(23)	29.0	150	
30	10	.05	29	35.6	17.7	17.9	101	+75
		.02	29	48.8	(31)	31.1	176	
26	12	.05	15	43.1	23.0	20.1	87	+56
		.02	15	55.9	(21)	32.9	143	
26	15	.05	14	45.7	23.0	22.7	99	+63
		.02	15	60.2	(21)	37.2	162	
28	15	.05	30	39.2	17.5	21.7	124	+66
		.02	30	50.8	(26)	33.3	190	
29	15	.05	30	38.1	19.3	18.8	97	+79
		.02	30	53.3	(23)	34.0	176	
25	30	.05	25	59.2	16.0	43.2	270	+53
		.02	25	67.6	(26)	51.6	323	
27	30	.05	24	49.1	18.5	30.6	165	+108
		.02	24	69.0	(28)	50.5	273	
25	50	.05	25	71.0	16.0	55.0	344	+43
		.02	25	77.9	(26)	61.9	387	
							Mean = 67.9%	
							$\sigma_M = \pm 5.4\%$	

*Figures in parentheses No. of chicks.

$$^1 \text{ Response as \% of } W_c = \frac{W - W_c}{W_c} \times 100$$

$$^2 \text{ Improvement (IR) as \% of } W_c = \frac{W_{.02} - W_{.05}}{W_c} \times 100$$

I, using mean comb weight for each group of chicks, regardless of sex, which had been treated in the same way. The dosages ranged from 10 to 50 γ . The response to treatment in any one experiment was measured by the increase in comb growth for that experiment (W) over the comb growth for a control experiment (W_c) run simultaneously, in which the chicks were untreated (columns 5 and 6). This increase (W-W_c), the comb weight corrected for controls, is given in column 7. It is apparent from the data that the response obtained in any one experiment with the smaller volume of vehicle is in each case greater than the response in the corresponding experi-

ment with the larger volume. Each of these response values has likewise been calculated as per cent of the corresponding control value and is given in column 8. The improvement in response resulting from reduction in volume of sesame oil (IR, column 9) is therefore measured by the difference of these paired percentage values. These "improvement" values are uniformly positive, corresponding to the greater response with 0.02 cc of oil than with 0.05 cc. They vary in magnitude from 43% to 108% of the corresponding control comb weight. The mean improvement is 67.9%, with a standard deviation of 5.4, and is based on a total of 710 chicks: 277 treated with the larger volume of oil, 278 with the smaller volume, and 155 untreated controls. A second group of experiments, identical with the foregoing but restricted to the dosage range 1-9 γ inclusive, has also been performed. This series employed a total of 308 chicks, of which 238 were treated and 70 were controls. The results were similar to those in the foregoing series.

It may be concluded, therefore, that this further reduction in volume of vehicle effects a further increase in the comb growth response of baby chicks. The only explanation which we can offer at the present time for this improvement is that the smaller the volume, the greater is the proportion of androgen solution utilized on the comb surface instead of being diverted to adjacent, less-sensitive head areas.

We desire to express our thanks to Dr. Erwin Schwenk of the Schering Corporation of New Jersey for supplying us with the androsterone used in this investigation.

11553

Improvement in Chick Comb Response to Androsterone Obtained with Alcohol as Vehicle.*

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In our studies of the bioassay method for androgens, utilizing the comb of the baby chick, it has appeared that further improve-

* This investigation was supported in part by a grant from the Friedsam Foundation.

ment in the method might be attained by utilizing some vehicle for the androgens other than sesame oil. We have been aware of the fact that an oily vehicle entails certain disadvantages: (1) It has a tendency to spread over the head feathers of the chick and thus cause a loss of variable amounts of the hormone by diversion from the comb. (2) As a solvent, oil is unsatisfactory particularly for the gummy residues frequently obtained in extracting androgens from urine. (3) The oil itself is not absorbed, as has been shown histologically by Soloway, Hansen and McCahey,¹ but the hormone is absorbed selectively by diffusion out of the vehicle instead of along with it. (4) The high viscosity of an oily vehicle is a distinct source of error in the preparation of quantitative solutions of the androgens. Other liquids which have been employed in place of sesame oil are tri-caproin (Hall and Dryden²), 60% alcohol for estrogens (Ito, Hajazu and Kon³), and 96% alcohol for testosterone (Zondek and Sulman⁴). The latter also mention the possibility of using benzol, ether, benzene and acetone but report no comparative studies with these solvents. Since 95% alcohol tends to overcome the undesirable features of sesame oil enumerated above, we have compared the two vehicles with respect to the comb growth response elicited by identical doses of androsterone. The procedure was similar to that described in the preceding paper (Klempner, Hollander and Frank⁵), wherein we compared the response elicited by the same dose of androsterone in paired experiments in which the only variable was the volume of sesame oil employed. In the present investigation, however, the paired experiments differed from each other in that the first employed 0.05 cc of sesame oil and the second 0.02 cc of 95% alcohol. A comparison of the relative efficiencies of the two liquids for the purpose of these investigations would have been simplified by the use of identical volumes, but—as will be shown below—this difference in volume can be canceled out of the final result without undue complications.

The data for 9 such pairs of experiments are presented in Table I. The response in any one experiment (column 7) is measured by the difference between the mean comb weight of the group of treated

¹ Soloway, D., Hansen, L. P., and McCahey, J. F., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 547.

² Hall, S. R., and Dryden, L. P., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **41**, 378.

³ Ito, M., Hajazu, S., and Kon, T., *Znbl. Gyn.*, 1937, **61**, 1094.

⁴ Zondek, B., and Sulman, F., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **40**, 633.

⁵ Klempner, E., Hollander, F., and Frank, R. T., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **44**, 631.

TABLE I.
Comparison of Alcohol and Sesame Oil as Vehicle in Bioassay of Androgens by
the Chick Comb Method.

Androgen dosage			No. of chicks	Mean comb wt		Response to androgen application (comb wt corrected for controls)		Improve- ment in response (IR) ²
No.	(γ)	Vehicle		Treated chicks	Control chicks	(W-W _c)	% of W _c ¹	
(1)	(2)	(3)		W (mg)	W _c (mg)	(7)	(8)	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
S39	10	Oil	18	48.1	23.6	24.5	104	+ 93
		Alcohol	17	70.2	(21)*	46.6	197	
S40	10	Oil	18	38.4	19.2	19.2	100	+155
		Alcohol	18	68.3	(37)	49.1	255	
S39	20	Oil	17	61.1	23.6	37.5	158	+152
		Alcohol	17	96.9	(21)	73.3	310	
S38	30	Oil	18	48.1	21.7	26.4	122	+182
		Alcohol	18	87.8	(25)	66.1	304	
S39	30	Oil	16	68.5	23.6	44.9	190	+134
		Alcohol	18	100.4	(21)	76.8	324	
S40	30	Oil	18	61.3	19.2	42.1	219	+172
		Alcohol	18	94.6	(37)	75.4	391	
S39	40	Oil	17	71.2	23.6	47.6	201	+167
		Alcohol	18	110.8	(21)	87.2	368	
S39	50	Oil	18	77.9	23.6	54.3	229	+206
		Alcohol	18	126.8	(21)	103.2	435	
S40	50	Oil	18	84.6	19.2	65.4	339	+140
		Alcohol	18	111.3	(37)	92.1	479	
							Mean = 155.7%	
							$\sigma_M = \pm 10.1\%$	

*Figures in parentheses are No. of chicks.

$$^1 \text{ Response as \% of } W_c = \frac{W - W_c}{W_c} \times 100$$

$$^2 \text{ Improvement (IR) as \% of } W_c = \frac{W_{alc} - W_{oil}}{W_c} \times 100$$

chicks (W, column 5) and that of the untreated control group in the same experiment (W_c, column 6). For comparative purposes, these "response" values are also given as per cent of the corresponding control weight (column 8). Then, the improvement in response obtained by the use of alcohol as compared with oil in any one pair of experiments is given by the difference between these percentage response values (IR, column 9). These "improvement" values are consistently in favor of the alcohol as vehicle, and vary from 93 to 206% of the corresponding control comb weight. The mean of these 9 IR values is 155.7% with a standard deviation of 10.1. This mean is based on a total of 538 chicks: 158 treated with oil, 160 treated with alcohol and 220 untreated controls.

It was mentioned above that the alcohol solutions were employed in daily volumes of 0.02 cc as compared with 0.05 cc for the sesame

oil. We have shown elsewhere (Klempner, Hollander and Frank⁵) that even with the same solvent such a difference in volume may produce a considerable increase in comb response; for 11 experiments with a range in dosage of 10-50 γ , the mean IR-value was $67.9 \pm 5.4\%$. However, in the present investigation the mean IR value is $155.7 \pm 10.1\%$. The difference between these two is 87.8% with a standard deviation of 11.5%. This difference is 7.6 times its standard deviation and is therefore statistically significant.

It may be concluded, therefore, that the improvement in comb growth response obtained in these experiments resulted from the substitution of alcohol for sesame oil, apart from the diminution in volume of vehicle. Such improvement may be ascribed to the rapid evaporation and absorption of the alcohol, with consequent diminution in loss by spreading to less responsive areas. It is also possible that the use of alcohol increases the rate of absorption of the androgen itself by the comb surface, but as yet we have no direct evidence of this.

We desire to express our thanks to Dr. Erwin Schwenk of the Schering Corporation of New Jersey for supplying us with the androsterone used in this investigation.

11554

Prevention of Nutritional Muscular Dystrophy in Suckling E-low Rats with Alpha-tocopherol and Related Substances.*

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That alpha-tocopherol was effective in preventing the dystrophy that appears in the suckling young of vitamin E-low mothers was first shown by Barrie,¹ Demole and Pfaltz,² and Goettsch and Ritzmann.³ Goettsch and Ritzmann found that FeCl₃-treated wheat

* Aided by grants from the Research Board and the Department of Agriculture of the University of California, the Rockefeller Foundation and Merck and Company, Rahway, New Jersey. The following materials were generously contributed: alpha-tocopherol by Merck and Company, Rahway, New Jersey. Assistance was rendered by the Works Progress Administration, Official Project No. 65-1-08-62, Unit A-5.

¹ Barrie, M. M. O., *Nature*, 1938, **142**, 799.

² Goettsch, M., and Ritzmann, J., *J. Nutr.*, 1939, **17**, 371.

³ Demole, V., and Pfaltz, H., *Schweiz. Med. Wochenschrift*, 1939, **69**, 123.

germ oil (which had no activity as vitamin E in the cure of sterility) likewise had anti-dystrophic activity.

We are able to confirm the findings of Barrie, Demole and Pfaltz, Goettsch and Ritzmann as to the anti-dystrophic activity of alpha-tocopherol.

Young female rats reared on our vitamin E-low diet 427⁴ and of proved sterility were bred for their second gestation and were given a single dose of 3 mg of natural alpha-tocopherol on the day of finding sperm. The litters resulting from these pregnancies were reduced to 6 young. In the first group of test animals the mothers were given 6 mg of alpha-tocopherol by stomach tube on the day of littering. They were allowed to suckle 3 of their own young and 3 foster young of the control mothers which received the solvent for the alpha-tocopherol (ethyl laurate). Likewise, the control rats were allowed to suckle 3 of their own young and 3 from the experimental animals. Six mg of alpha-tocopherol was almost adequate to prevent the dystrophy that would otherwise have developed toward the end of the lactation period. Two animals, however, in this group exhibited a slight stiffness (Table I). Ten mg of alpha-tocopherol appeared to be adequate to prevent the dystrophy.

TABLE I.
Prevention of Muscular Dystrophy in Suckling E-low Rats with α -tocopherol.

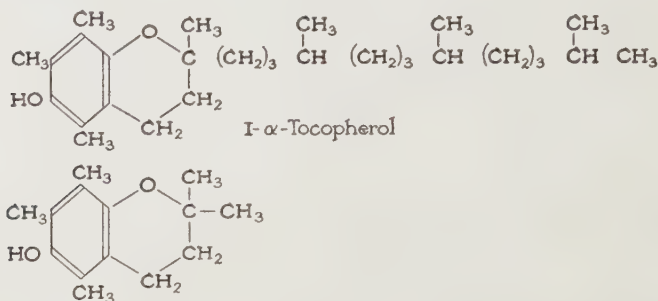
Treatment	No. of young	Dystrophic or dead Days 15-25
Mother received 6 mg α -tocopherol (in ethyl laurate) on day of littering	30	2 (slight stiffness)
Mother received ethyl laurate only	64	64
Mother received 10 mg α -tocopherol on day of littering	41	0
Young received 1 mg α -tocopherol daily from day 10	13	0
Young received ethyl laurate only	12	6
Young received 3 mg α -tocopherol daily from day 15	12	0
Young received ethyl laurate only	9	6
Young received 3 mg α -tocopherol daily from day 18	30	25
Young received ethyl laurate only	25	19

In a second experiment the young were given alpha-tocopherol: one group received 1 mg daily from day 10; a second group received 3 mg daily from day 15; and a third group, 3 mg daily from day 18. The results of this experiment demonstrate that alpha-tocopherol when administered as late as day 15 of lactation was effective in preventing the paralysis but that the young receiving the alpha-tocopherol from day 18 were not protected. It can be seen that not all the ethyl laurate treated young developed dystrophy although this was the case when the mothers were so treated. An explanation

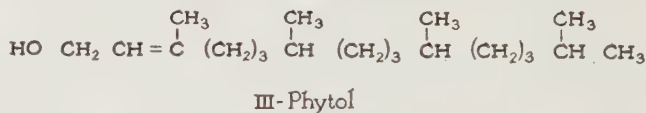
⁴ Emerson, G. A., and Evans, H. M., *J. Nutr.*, 1937, **14**, 169.

may be afforded by the necessary conditions of the experiment for young in the same litter were fed the alpha-tocopherol by dropper and slight oral residues could have been licked off by litter mates.

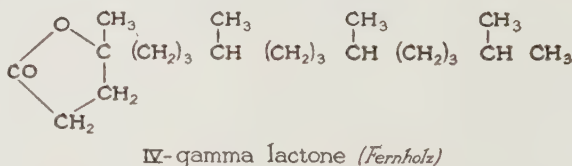
Following the observation of Goettsch and Ritzmann demonstrating the activity of FeCl_3 -treated wheat germ oil in preventing the dystrophy, we fed substances related chemically to alpha-tocopherol (I).



II-2,2,5,7,8 pentamethyl 6 hydroxy chromane has the same ring structure as alpha-tocopherol. The side chain of alpha-tocopherol is derived from phytol (III),

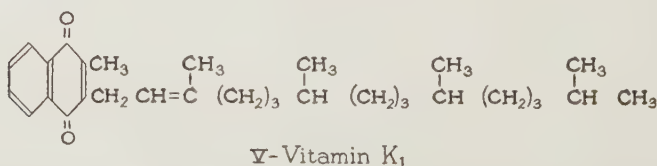


Fernholz obtained the gamma lactone (IV)



on oxidation of alpha-tocopherol. All of these substances were inactive whether fed to the young from day 15 or to the mothers on the day of littering (Table II).

The resemblance in structure between vitamin K_1 (V)



and alpha-tocopherol suggested that this substance might act as vitamin E but vitamin K_1 was also found to be inactive in the prevention of muscular dystrophy.

TABLE II.
Failure to Prevent Muscular Dystrophy in Suckling E-low Rats with Substances
Related to α -tocopherol

Treatment	No. of young	Dystrophic or dead Days 15-25
Young received 3 mg phytol daily from day 15	16	14
Young received 3 mg 2,2,5,7,8, penta methyl 6 hydroxy chromane daily from day 15	20	13
Young received 3 mg gamma lactone (Fernholz) from day 15	16	13
Young received ethyl laurate only	52	45
Mother received 15 mg 2,2,5,7,8 penta methyl 6 hydroxy chromane on day of littering	54	53
Mother received 15 mg vitamin K ₁ on day of littering	18	17
Mother received ethyl laurate only	28	26
Mother received 15 mg gamma lactone (Fernholz) from day 15	24	24

Summary. The dystrophy that almost invariably appears toward the end of the lactation period in the suckling young of vitamin E-low mothers can be prevented by the administration of 10 mg of alpha-tocopherol to the mother on the day of littering or the feeding of 1 mg daily to the young from day 10 or 3 mg from day 15. The administration of 3 mg of alpha-tocopherol daily from day 18 was ineffective. The following compounds related chemically to alpha-tocopherol were tested for anti-dystrophic activity and found inactive: 2,2,5,7,8-penta-methyl, 6 hydroxy chromane, phytol, gamma lactone and vitamin K₁.

11555 P

Reimplantation and Transplantation of Eyes in Anuran Larvae and *Fundulus heteroclitus*.*

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These experiments were undertaken on anurans and fishes to compare the results with those obtained from a series of studies on the grafted eyes of urodeles.¹⁻⁵ In one group the functional eye in

* Aided by grants from the John and Mary R. Markle Foundation and the Fluid Research Fund of Yale University School of Medicine.

1 Stone, L. S., *J. Exp. Zool.*, 1930, **55**, 193.

2 Stone, L. S., and Cole, C. H., *PROC. SOC. EXP. BIOL. AND MED.*, 1931, **29**, 176.

3 Stone, L. S., Zaur, I. S., and Farthing, T. E., *PROC. SOC. EXP. BIOL. AND MED.*, 1934, **31**, 1082.

4 Stone, L. S., Ussher, N. T., and Beers, D. N., *J. Exp. Zool.*, 1937, **77**, 13.

5 Stone, L. S., and Chace, R. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1937, **36**, 830.

Fundulus heteroclitus, 4 to 6 cm in length, was grafted in the orbit (32 reimplants and 20 transplants). For several days, or in some cases for weeks, the operated eye appeared perfectly normal. Circulation in most cases returned in 2 or 3 days and ocular movement was present in 10 days. The lens in some cases was slightly opaque as early as the tenth day. It usually broke down rapidly and in the living eye during the first or second month it appeared as a white gelatinous mass protruding through the pupil.

At the end of a month the pupil and the eye became slightly smaller. The iris began to show pigment changes and during the second month most eyes were slowly resorbed. Throughout the experiment the cornea never became opaque.

The animals were sacrificed 1 to 95 days after operation. Histological sections showed that the central region of the retina degenerated rapidly, beginning on the second day. The rod and cone cells in this area were slightly more resistant than other layers. The ciliary region, so resistant in urodeles (opus cited), was still a complete ring of cells on the third and fourth weeks when the rest of the retina was a mass of débris. In one unusual case (2 reimplanted eyes on the same host) both eyes on the ninety-fifth day appeared much like the normal. The lenses were clear and undegenerated and the retina had not undergone extensive degeneration.

Eighty-two eyes were reimplanted in the orbit in *Rana pipiens* larvæ 18 mm in length. Thirty eyes sloughed out in 24 hours after operation. Fifty-two healed in place and circulation returned as early as the second day. Forty-three specimens were sacrificed from one to 96 days after operation for histological studies. Several were preserved one month after metamorphosis. The growth and size of many eyes equalled the normal while some were slightly smaller. Ocular movement was observed as early as the tenth day. The cornea was never opaque and in only 2 instances (specimens killed on the third and fourth days) was the lens cloudy. Histological studies showed that in the region of the optic nerve the retina possessed varying amounts of degenerating cells during the first week. A number of cells were also lost throughout the ganglion cell layer. Some cells were lost locally in the inner nuclear zone while the rod and cone cells seemed to be normal. During the second week the optic nerve degenerated as far as the chiasma. From the second week to one month after metamorphosis there was no further sign of degeneration. If injuries were not extensive at operation the retina healed early but always carried the scar. These eyes showed very little capacity to regenerate even an optic nerve. This is quite different from the results obtained in urodeles (opus cited).

Ten eyes were reimplanted in the orbit in *Rana clamitans* larvæ about 65 mm in length. Three died immediately after operation. Two eyes were severely injured at operation and were slowly resorbed by one month before the hosts metamorphosed. Five cases were sacrificed from 150 to 227 days after operation. Two of these were carried to metamorphosis and 3 were killed from 53 to 80 days after metamorphosis. All eyes and their pupils were slightly smaller than normal at the end of the experiment.

Histological studies showed that no degeneration was taking place in the eye when the hosts were killed. All retinae were slightly thinner than normal. They showed a reduction in the number of ganglion cells and the inner nuclear zone was not as deep as the normal. In every case the small optic nerve stump at the bulb did not penetrate further than the choroid coat. The optic nerve was completely absent from the bulb to the chiasma. The lens was slightly smaller but normal histologically. All other structures appeared normal in both the living state and in histological preparations. The results in these older larvæ seem to be about the same as in those of the much younger *R. pipiens*.

11556

Occurrence of Riboflavin in Tubercle Bacillus.*

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It has been known for several years that aqueous extracts of the tubercle bacillus exhibit yellow fluorescence in ordinary light. We have attempted the purification and characterization of this yellow pigment. Extracts of partly defatted tubercle bacilli made with 25% alcohol showed a yellow fluorescence in ordinary light and a beautiful blue fluorescence in ultraviolet light. The formation of lumiflavin upon alkaline irradiation indicated the presence of a

* Aided by grants from the Research Committee of the National Tuberculosis Association and from the Rockefeller Foundation.

† Vitamin Research Fellow at Yale University, 1936-38.

‡ Holder of a National Tuberculosis Association Fellowship at Yale University, 1936-37.

flavin-like pigment. This pigment was partially purified, as described below, and fed to rats receiving a diet lacking riboflavin. The resulting growth response indicated that the bacterial flavin was the well known water-soluble vitamin, riboflavin.¹

At the time this work was done in 1937, there were no published reports indicating the chemical nature of this yellow pigment of the tubercle bacillus. However, since that time, Boissevain, Drea and Schultz² have announced the isolation of riboflavin from the tubercle bacillus, identified by melting point and absorption spectrum. Our work on the biological activity of the pigment may be considered to confirm its identity as riboflavin.

The several strains of tubercle bacilli used in this investigation had been grown on the Long³ Synthetic Medium in the Biological Laboratories, Sharp and Dohme, at Glenolden, Pennsylvania. The material represented the dried cell residues after extraction with alcohol, ether and chloroform.

The cell residues from tubercle bacilli, Strain A-14, had been extracted in 1934 as described by Crowder, Stodola, Pangborn, and Anderson.⁴ For the present examination 522 g of the dried cell residues were digested and extracted repeatedly with 2-liter portions of warm 25% alcohol. The combined extracts were filtered through a Chamberland filter and the clear filtrate was concentrated *in vacuo* to a volume of 1076 cc. This extract contained 54.8 g of solids, which were mostly polysaccharides and inorganic salts, but a small amount of protein was present, together with other undetermined constituents.

The extract showed a yellowish-green fluorescence in ultraviolet light. At pH 2 the fluorescence was green, but in the range of pH 3 to 5 it was blue when irradiated with ultraviolet light. Extraction with chloroform or other solvents either at neutral, acid or alkaline reaction did not remove any fluorescent pigment.

The amount of flavin was estimated in 2 cc of the extract by conversion to lumiflavin by irradiation in alkaline solution according to the procedure of Warburg and Christian.⁵ The lumiflavin thus produced was extracted by chloroform from the acidified solution and estimated by means of the Pulfrich spectrophotometer according

¹ Kuhn, György, and Wagner-Jauregg, *Ber.*, 1933, **66**, 576, 1034.

² Boissevain, Drea, and Schultz, *Proc. Soc. Exp. Biol. and Med.*, 1938, **39**, 481.

³ Long, *Am. Rev. Tuberc.*, 1926, **13**, 393.

⁴ Crowder, Stodola, Pangborn, and Anderson, *J. Am. Chem. Soc.*, 1936, **58**, 636.

⁵ Warburg and Christian, *Biochem. Z.*, 1933, **266**, 377.

to Kuhn, Wagner-Jauregg and Kaltschmitt.⁶ On this basis, it was calculated that the original dry bacilli contained 36.6 mg of flavin per kilo.

Portions of dry defatted human strain A-12 and the avian tubercle bacillus, Hygienic Laboratory No. 531, both grown on the Long³ synthetic medium, were also examined for flavin pigments. The extraction and the estimation as lumiflavin were carried out as above. The values found corresponded to 13.0 mg of flavin in the human strain A-12 and 19.3 mg in the avian bacilli per kilo of original dry bacteria. These values can only be regarded as minimal. Living bacilli would undoubtedly contain larger quantities of flavin.

Concentration of Flavin. A considerable amount of polysaccharide and other solid matter was removed from the aqueous solution of the A-14 extract by precipitation with three parts acetone and one part alcohol. This precipitate also contained much of the nonflavin fluorescing pigments which have not yet been adequately investigated.

The supernatant solution was concentrated to 800 cc, adjusted to 0.1 N with H₂SO₄ and the flavin was adsorbed by shaking with fuller's earth. After elution with pyridine-methanol-water the concentration was continued by formation of the silver salt in the usual manner. Removal of the silver left 0.487 g of solid material containing 2 mg of flavin, determined by the irradiation procedure.

Biological Assay. The crude flavin concentrate was assayed as follows: A litter of eight 21-day-old albino rats were fed *ad libitum* a basal diet, complete except for the vitamin B complex, of the following composition: casein 18, sucrose 73, salt mixture, Osborne and Mendel, 4, Crisco 2, and cod liver oil 3 parts. In addition, each rat received daily a rice polish extract (tikitiki)⁷ equivalent to 1 g of rice polish. We have shown⁷ that this tikitiki contains all the members of the vitamin B complex required by rats except riboflavin, of which only traces are present.

When growth had nearly ceased, one group of rats was fed the bacterial flavin, one group was given crystalline riboflavin,[§] and a third group was continued on the diet without further supplement. The growth curves are shown in Fig. 1 and indicate that the bacterial flavin possessed the same biological activity as crystalline riboflavin.

⁶ Kuhn, Wagner-Jauregg, and Kaltschmitt, *Ber.*, 1934, **67**, 1452.

⁷ Street and Cowgill, *Am. J. Physiol.*, 1939, **125**, 323.

[§] Obtained from the Borden Company, Bainbridge, N. Y., as crystalline lacto-flavin, P X grade.

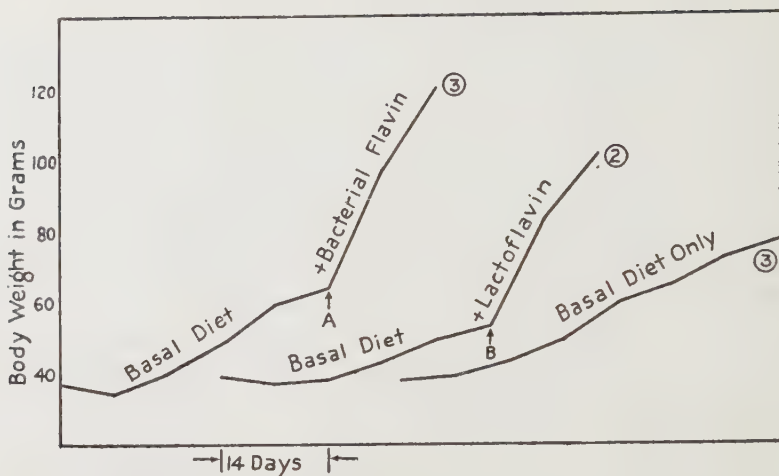


FIG. 1.

The effect of bacterial flavin and of crystalline riboflavin on the growth of rats fed a basal diet supplemented with rice polish extract. At point A the daily administration of the bacterial flavin preparation in amount calculated to supply 40 γ of flavin was begun. At point B the daily administration of 40 γ of pure crystalline riboflavin was begun. The curves are averages. The figure in the circle by each curve represents the number of animals in the group.

In view of the uniformity in growth response of the several animals, it appears justifiable to present the results in the form of curves based on averages. While the number of animals used was necessarily small, many other tests performed in this laboratory using the same basal diet, including rice polish extract alone or supplemented with crystalline riboflavin, have yielded results practically identical with the curves shown above.

The average daily gain in weight of 3.5 g daily observed in these experiments with a daily supplement of 40 γ of riboflavin, is essentially normal growth for this strain of rats and indicates that the basal ration supplies adequate amounts of all nutritional essentials required by the rat other than riboflavin. Under these circumstances, any increased gain over that of the negative controls produced by a vitamin bearing substance should be specific for riboflavin. Since this work was completed one of us (H.R.S.) has used a ration, containing tikitiki as a source of the B complex, for riboflavin assays involving the use of over 1000 rats, with entirely satisfactory results.

Summary. The tubercle bacillus cultivated on the Long synthetic medium is capable of synthesizing fluorescent flavin pigment. This pigment, when fed to young rats, promotes growth to the same extent as a corresponding quantity of crystalline riboflavin.

11557

Effect of Certain Purines, and CO₂ on Growth of Strain of Group A Hemolytic Streptococcus.*

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Rapid luxuriant growth of the C203S strain of Group A hemolytic streptococcus, equal to that occurring in broth under comparable conditions, has been obtained on a medium of essentially known composition. The complete medium is made up as follows:

I. 40 cc of stock solution of acid hydrolyzed gelatin equivalent to 25% gelatin¹ are diluted with distilled water to 500 cc and 500 mg cystine dissolved in a few cc of dilute HCl, 3 g KH₂PO₄, 1 g Na₂HPO₄ (anhydrous), and enough 5N NaOH to bring the pH to 7.4-7.6 are added. The solution is boiled gently for 5 minutes and filtered.

II. To the filtrate are added 50 mg tryptophane, 100 mg tyrosine, 15 mg adenine sulfate, 10 mg uracil, 0.2 mg nicotinic acid, 2 mg synthetic vitamin B₆,[†] 0.1 mg of biotin concentrate[‡] and 2 cc of salt mixture (25 g MgSO₄ · 7 H₂O, 20 mg MnCl₂ · 4 H₂O, 5 mg CuSO₄, 2 mg FeSO₄ · 7 H₂O, and 2 mg ZnSO₄ · 7 H₂O made up to 100 cc with water containing a few drops of concentrated HCl). The volume is made up to 900 cc, the solution readjusted to pH 7.4-7.6, tubed in 9 cc amounts and autoclaved at 10 lb for 10 minutes.

III. To each tube is added 0.1 cc of the following solutions which have been sterilized separately: 0.1 mg thiamin (vitamin B₁) per cc, 0.05 mg riboflavin per cc, 0.1 mg synthetic d-calcium pantothenate per cc, 1% neutralized thioglycollic acid containing 0.2 mg glutathione per cc and 5 mg glutamine per cc. The last three solutions

* The expenses of this work have been defrayed by a generous grant from the Commonwealth Fund.

¹ Pappenheimer, A. M., Jr., and Johnson, S. J., *Brit. J. Exp. Path.*, 1937, **18**, 239.

[†] We are greatly indebted to Merck & Co. for generous samples of synthetic vitamin B₆ and calcium pantothenate.

[‡] We are indebted to Dr. D. W. Woolley of the Rockefeller Institute, New York, for this concentrate which was prepared according to the procedure of Woolley, D. W., McDaniel, L. E., and Peterson, W. H., *J. Biol. Chem.*, 1939, **131**, 381. From its activity in promoting growth of *Clostridium butylicum* Dr. Woolley estimates that approximately 4% of the material is actual biotin. (See also Peterson, W. H., McDaniel, L. E., and McCoy, E., *J. Biol. Chem.*, 1940, **133**, lxxv.)

are sterilized by filtration. Finally, 0.5 cc of 5% glucose containing 0.04% CaCl₂ · 2 H₂O is added to each tube.

IV. The inoculum consists of one drop of 6-8-hour broth culture of C203S which has been twice washed with saline and then made up to slight turbidity with saline. Tubes are incubated under 8 mm CO₂ tension in air for 40 hours. The amount of growth is determined with a photoelectric colorimeter and the readings correlated with standard suspensions of known bacterial nitrogen content.

The effect of omitting each of the above factors in turn from the complete medium is listed in Table I. Figures in the second column indicate the minimum amount of substance necessary for optimum growth in 10 cc of medium.

All the substances listed in Table I are essential for rapid growth with the exception of uracil. Uracil, while not essential, appears to increase growth slightly.

In the complete medium the limiting factor appears to be glucose. Addition of more glucose will increase growth proportionately until sufficient acid has been produced to kill the organisms.

The need for pantothenic acid and riboflavin agrees with the results of Rane and Subbarow,²⁻³ McIlwain,⁴ and Woolley and Hutchings⁵ using other strains. The necessity of glutamine for rapid growth confirms the work of McIlwain *et al.*⁶ In agreement with McIlwain we have found that glutathione is not essential provided the thioglycollic acid concentration is increased to 10⁻³ molar in the final medium. Our findings with respect to vitamin B₆ would also seem to confirm those of McIlwain and of Woolley and Hutchings⁵ on Group D strains. Thiamin has not previously been reported essential for growth of hemolytic streptococcus. While it does not seem improbable that biotin is necessary for growth of the C203S strain, this cannot be regarded as certain until pure biotin becomes available for test. It is also possible that some other essential factor may be present as impurity in the gelatin or in the glutamine, both of which were prepared from natural sources. This seems unlikely in view of the good growth obtained.

We have been particularly interested in the requirement of strain

² Rane, L., and Subbarow, Y., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 837.

³ Subbarow, Y., and Rane, L., *J. Am. Chem. Soc.*, 1939, **61**, 1616.

⁴ McIlwain, H., *Brit. J. Exp. Path.*, 1939, **20**, 330; *Brit. J. Exp. Path.*, 1940, **21**, 25.

⁵ Woolley, D. W., and Hutchings, B. L., *J. Bact.*, 1939, **38**, 285.

⁶ McIlwain, H., Fildes, P., Gladstone, G. P., and Knight, B. C. J. G., *Biochem. J.*, 1939, **33**, 223.

TABLE I.

Effect of Omitting Certain Factors from Complete Medium on Growth of C203S Strain.

Substance	Amt necessary for optimal growth		Mg bacterial N after 40 hr incubation from 10 cc × 50
Complete medium	—		10-13
No glucose	25	mg	0.1
" glutamine	500	μg	variable*
" tyrosine	1000	"	3.9
" tryptophane	500	"	0.8
" uracil	100	"	9.9
" adenylic acid (0.4 mm CO ₂ tension)	100	"	0.7
" CO ₂ (CO ₂ -free air)	8	mm	2.2
" thioglycollic acid	1	mg	} 0.1
" glutathione	4	μg	
" thiamin	0.01	"	3.1
" nicotinic acid	1.0	"	1.9
" pantothenic acid	10	"	0.2
" riboflavin	0.04	"	0.8
" vitamin B ₆	20	"	5.9-7.1†
" "biotin" concentrate	1.0	"	1.8
Broth (25 mg glucose in 10 cc)	—		10-17

*No significant growth at 20 hours without glutamine. Growth at 40 hours at 8 mm CO₂ tension is variable.

†No significant growth at atmospheric CO₂ tension without vitamin B₆.

C203S for adenine or related compounds. If purine is omitted from the complete medium no growth occurs within 40 hours. Addition of adenine permits growth to occur. Adenine may be replaced by adenosine or adenylic acid, by guanine, guanosine or guanylic acid and by xanthine or hypoxanthine. It cannot be replaced by uric acid, caffeine or theophylline or by the pyrimidines uracil and cytosine. Subbarow and Rane³ have reported that certain of the above purines "may be of significance" in the growth of the N.Y. No. 5 strain of hemolytic streptococcus. McIlwain⁴ has included a number of purines in his medium on general grounds and Möller⁷ has shown that adenine or guanine but not xanthine or hypoxanthine increase the growth of *Lactobacillus plantarum*.

The purine requirement of strain C203S was discovered before all of the other growth factors had been identified. Upon reexamining the purine requirements using the more defined medium given in detail above, it was noted that the presence of 5% carbon dioxide in the atmosphere above the culture greatly accelerated growth and the surprising observation was made that even when purine was absent growth occurred. We therefore examined the effect of carbon dioxide in some detail and in Table II are shown some of the

⁷ Möller, E. F., *Z. Physiol. Chem.*, 1939, **260**, 246.

TABLE II.
Effect of Adenylic Acid on Growth of Strain C203S at Different Carbon Dioxide Tensions.

Carbon dioxide tension (mm Hg)	No adenylic acid		Adenylic acid, 10 µg/cc	
	20 hr	40 hr	20 hr	40 hr
CO ₂ -free air	—	0.3	—	2.2
0.4	0.0	0.7	1.9	6.8-8.2*
1.4	0.1	0.7	3.1	8.4
2.4	0.15	3.8	3.0	9.1
4.3	0.15	9.0	10.9	10.1
8	1.4	9.1	11.3	10.4
20	1.0	9.2	9.6	10.8
40	10.1	12.2	12.2	12.4
8 mm CO ₂ , 730 mm nitrogen, no oxygen	—	12.5	—	12.5

*Growth is quite variable at atmospheric CO₂ tension which has been assumed to be 0.4 mm Hg.

Note that readings at 20 and 40 hours were from different experiments in different tubes. Growth is given as milligrams bacterial nitrogen per 10 cc × 50. Figures represent averages of at least 2 tubes.

The oxygen tension was kept constant at 120 mm throughout except in the anaerobic experiments and those done at atmospheric CO₂ tensions. The gas pressure was made up to 740 mm with nitrogen in each case.

results at different CO₂ tensions with and without adenylic acid. It will be noted that maximal growth when adenylic acid is present occurs within 20 hours provided the CO₂ tension is 4 mm or greater, that no significant growth occurs even after 40 hours' incubation in the absence of adenylic acid when the CO₂ tension is below 2 mm and that even when the CO₂ tension is high, a small but consistent increase in growth is apparent at 40 hours when adenylic acid is added to the medium. It has been observed that the bicarbonate ion cannot replace carbon dioxide when no purine is present. However, in the presence of both adenylic acid and bicarbonate, any slight growth of the organisms may liberate sufficient carbon dioxide through action of the acid produced to accelerate growth. These observations on the accelerating effect of carbon dioxide are in harmony with those of other workers⁸ and indeed McIlwain⁴ grew his cultures in 5% carbon dioxide.

At present we have no clue as to the significance of these findings. Whether carbon dioxide is necessary for purine synthesis or whether purine plays a rôle in the production of carbon dioxide by the organisms cannot be decided at this time. We may point out, however, that a somewhat analogous situation has been reported in the case of *Staphylococcus aureus* by Richardson.⁹ With this organism no

⁸ Gladstone, G. P., Fildes, P., and Richardson, G. H., *Brit. J. Exp. Path.*, 1935, **16**, 335.

⁹ Richardson, G. H., *Biochem. J.*, 1936, **30**, 2184.

growth occurs under anaerobic conditions unless uracil is added. In the presence of oxygen uracil is non-essential.

The C203S strain of streptococcus is a powerful hemolytic strain. In our experience hemolytic titers on this medium are equivalent to those obtained in broth, provided care is taken to avoid accumulation of acid during growth. The growth and hemolysin titer may be increased by addition of more glucose to the medium and periodic neutralization of the acid formed. However, preliminary work in this direction indicates that some factor, as yet unidentified, becomes the limiting one under these conditions.

Summary. Rapid, heavy growth and hemolysin production of the C203S strain of Group A hemolytic streptococcus have been obtained on a medium of essentially known composition. In addition to factors reported by previous workers, we have found that thiamin, nicotinic acid, adenine or related purines, and an unknown factor which may possibly be biotin are necessary for growth of this strain. The relation of carbon dioxide tension to the purine requirement has been studied.

11558

Modifying Influence of Light on Chick's Comb Response to Androsterone.

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(Introduced by S. R. Haythorn.)

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The assay of androgenic material by biologic methods offers definite advantages over colorimetric determinations when we consider that in measuring the 17 ketosteroids by chromogenic effect we are determining both biologically active and inactive material.¹ In many cases the important consideration is, to what degree is biological activity present in a given specimen. In consequence of this, we made comparisons of colorimetric² and biologic determinations in some of our studies. The comb response of one-day-old

¹ Callow, N. H., Callow, R. K., Emmens, C. W., and Stroud, S. W., *J. Endo.*, 1939, **1**, 76.

² Neustadt, Rudolph, *Endo.*, 1938, **23**, 711.

chicks, previously reported by Dorfman and Greulich,³ Emmens,⁴ and Frank and Klempner,⁵ was employed as the method of studying biologic response. During the preliminary plotting of our curves, to establish normal average response to known dosage, it was recognized by us that light played an extremely important rôle as a modifying factor in the weight response of the chick's comb. Though this factor has been observed in the capon,⁶ we have found no mention in the literature regarding the baby chick. It is, therefore, of paramount importance that the influence of light be recognized and taken into account by workers who have adopted the chick comb weight method for the assaying of androgens.

The 309 white leghorn male chicks used in our experiment were procured from the same hatchery on the day of hatching. All chicks used were hatched during the first 3 weeks of April. When the chicks were one day old, daily inunction of the combs was begun and continued for 6 days. The combs were removed and weighed on the day following cessation of treatment and the weights of the birds also were noted. Removal of the comb was accomplished by making lateral and posterior incisions at the base of the comb of the anesthetized chicks and by grasping the anterior end with forceps and stripping the comb off dorsally. All combs were removed by the same individual.

Daily treatment consisted of spreading 0.01 cc of sesame oil over the surface of the comb. This was easily accomplished with accuracy by the use of a micro-titrating pipette calibrated in 0.002 cc divisions and which had the tip bent at a right angle. This amount of oil contained from 0.5 to 50.0 γ of androsterone* and was applied at each inunction to groups of from 5 to 14 chicks. The doses used in the various groups are indicated in Fig. 2 and 3. The oil applied was absorbed within about 10 minutes, during which period the chicks on each dose were kept separated to prevent contamination through contact. Control animals which received only sesame oil were kept in separate cages.

The chicks were divided into 4 groups, each group being exposed to varying degrees of light and darkness. One hundred and five

³ Dorfman, R. I., and Greulich, W. W., *Yale J. Biol. and Med.*, 1937, **10**, 79.

⁴ Emmens, C. W., Med. Res. Council "Reports on Biological Standards—V," 1939.

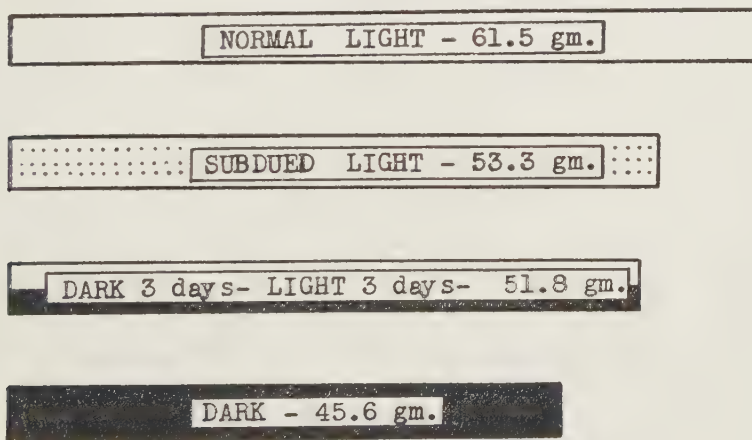
⁵ Frank, R. T., and Klempner, E., *Proc. Soc. Exp. Biol. and Med.*, 1937, **36**, 763.

⁶ Womack, E. B., Koch, F. C., Domm, L. V., and Juhn, M., *J. Pharm. Exp. Therap.*, 1931, **41**, 173.

* The androsterone in this study was kindly supplied to us by Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products, Inc.

animals were maintained in a large incubator in total darkness, being brought into daylight only during the period of treatment which did not exceed one hour per day. Thirty-three chicks were placed in a room where they received very little natural light, referred to as "subdued". Ninety-one animals were placed in a large, well-lighted, airy room where both natural and artificial light was adequate and in which the cage was so located that all the chicks received approximately the same amount of light. The last group of 100 chicks was placed in total darkness for the first 3 days of treatment and were then placed in the same room as the chicks of Group 3 for the second 3 days. Although a mortality of approximately 50% was noted among the groups kept under deficient lighting conditions, only 5% died when the animals were kept in adequate light. The numbers of animals referred to above represent the surviving members of each group.

The effect of the various light conditions may be seen in Fig. 1 where the average body weights of each group, taken at the seventh day, have been presented. The inhibition to body growth, due presumably to inadequate light, is clearly seen where a direct correlation between the amount of light the chick received and the body weight is made. The influence of light alone on the body growth of chicks had been previously reported.⁷ The body weights of each



AVERAGE BODY WEIGHTS OF CHICKS
UNDER VARIOUS CONDITIONS OF LIGHT

FIG. 1.

⁷ Bovie, W. T., *Boston Med. and Surg. J.*, 1925, **192**, 1035.

of our groups were plotted against the dosage of androsterone but failed to indicate any correlation with the treatment. This graph has been omitted.

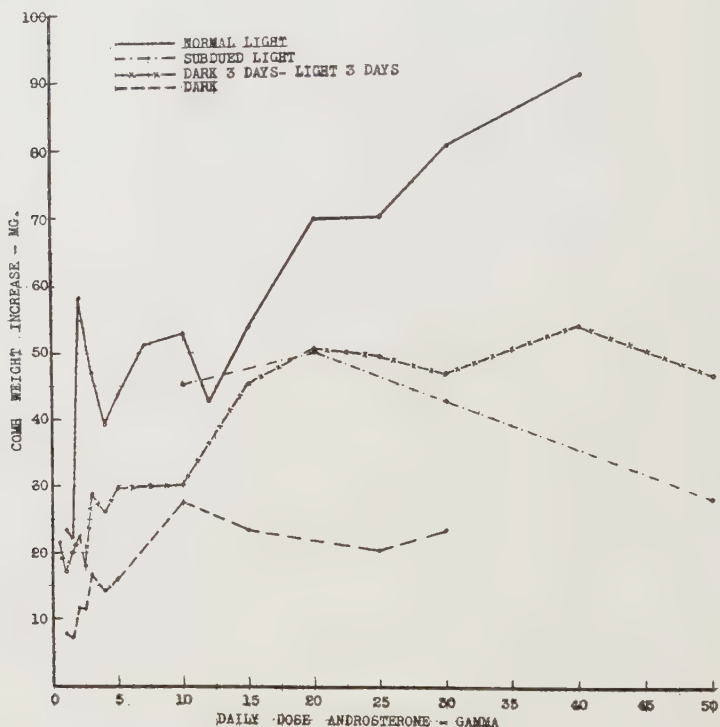


FIG. 2.

Variations in Comb Weight Increase as Influenced by Altered Light Intensity.

In Fig. 2, the average comb weight increase of the treated animals over the control comb weights has been plotted against the daily dosage of androsterone. A comparison of the curves in this figure shows the effect of exposing the chicks to varying degrees of light intensity while employing the same dose of androsterone. There is a slight response of the animals maintained in the dark as compared with those in normal light, with the groups exposed to inadequate light occupying a median position. It is also of interest to observe that the animals kept under deficient light conditions failed to show a corresponding comb weight increase with the application of higher concentrations of androsterone as did the groups in normal light. The curves in the "deficient light groups" tend to reach a plateau. The curve of the groups in normal light is still fairly steep at a daily dosage of 40 γ , a fact rather important if this method is to be considered for assay of unknown extracts.

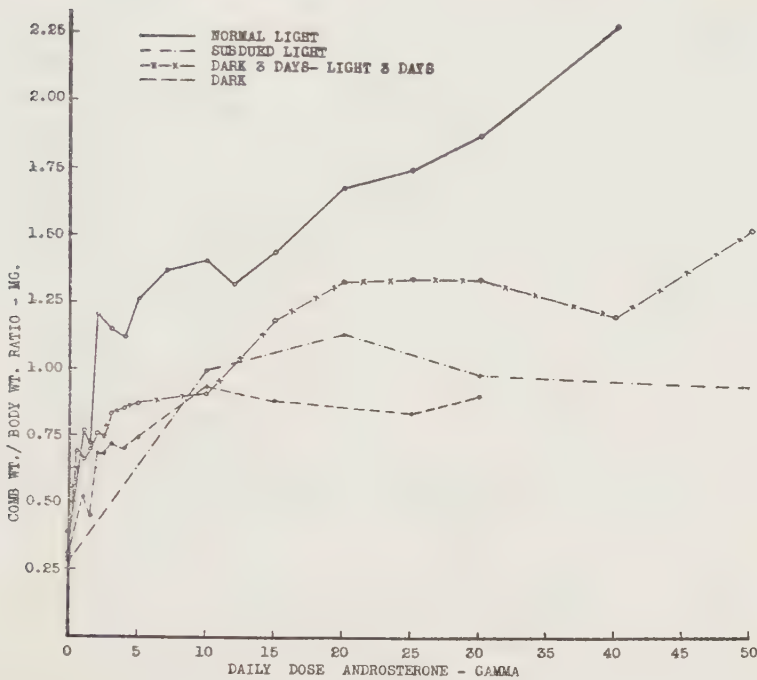


FIG. 3.

Comb Weight Body Weight Ratio Curve as Influenced by Altered Light Intensity.

In Fig. 3, the ratios of average comb weight over the average body weight have been plotted against the daily dose. Consideration of the comb weight in relation to body weight tends to remove some of the variations occurring when the comb weights alone are considered.

It becomes evident that two variable factors influence the growth of the combs of chicks; namely, the amount of androgenic material employed, and the intensity of the light to which the chicks are exposed. The light factor must, therefore, be made constant and of adequate intensity. With the changing seasons of the year, this must be borne in mind and due corrections made if a constant response is to be secured and sources of error are to be reduced to a minimum. Disregard of the amount of light available to the chicks may easily explain the discrepancies in results obtained by various workers. It is not within the province of this report to postulate whether the stimulating influence of androsterone is inhibited by the lack of light, but it is evident there is sufficient physiological inhibition to the animal organism so that it acts as a factor in preventing proper response.

Summary. Exposure to varying degrees of light influenced the

weight response of male chicks' combs to which androsterone had been applied by inunction. The weight response of the combs to androgenic stimulation is considerably greater in birds receiving normal daylight than in those kept in the dark or in inadequate light. Body weights of the various groups indicate a lack of growth which is in direct correlation to the lack of light. The importance of exposing the test birds to an adequate and a constant source of light is emphasized if this method is to be used for the assay of unknown androgens.

11559

Renal Physiology in Infants and Children: I. Method for Estimation of Glomerular Filtration Rate.

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The more exact methods for estimation of kidney function have been quite successfully employed in older children,¹ but they have not been very widely applicable to infants because of the obvious difficulties in obtaining complete urine specimens. Schoenthal and his coworkers² studied the urea clearance in 9 normal infants ranging in age from 2 to 11½ months and concluded that the urea clearance corrected for surface area agreed with the values observed by Van Slyke and his coworkers for older children and adults, which indicated to them that renal function measured by the ability to excrete urea is as well developed in infants as in later life. Their studies did not include, however, the investigation of renal function during very early postnatal life. The method to be described here originated in an attempt to study the renal physiology during the newborn period. The single injection inulin clearance test proposed by Alving and Miller³ seemed especially applicable to this problem. Inulin clearances seemed preferable to urea clearances for this study since it has been well established that the inulin clearance is at the

¹ Cullen, G. E., Nelson, W. E., and Holmes, F. E., *J. Clin. Invest.*, 1935, **14**, 563.

² Schoenthal, L., Lurie, D., and Kelly, M., *Am. J. Dis. Child.*, 1933, **45**, 41.

³ Alving, A. S., and Miller, B. F., *A Practical Method for the Measurement of Glomerular Filtration Rate (Inulin Clearance)*, to be published.

level of glomerular filtration and that the filtration rate is fairly constant at low and high rates of urine formation.⁴

Alving and Miller have shown that after the intravenous injection of inulin (10 g in adults) the plasma inulin concentration, plotted logarithmically against time, decreased at first in a curvilinear manner, later falling, however, in a linear or almost linear relationship with time. The curvilinear relationship marks, presumably, the period of equilibration between the blood and plasma and the extracellular fluids. The straight line relationship follows because once equilibrium has been established the rate of fall of the plasma inulin level is determined by the rate at which inulin is cleared from the plasma by the kidneys. Since inulin is quantitatively excreted in the urine after intravenous injection, and since, as previously stated, the inulin clearance is independent of the rate of urine formation, the rate of fall of the inulin in the plasma after the straight line relationship has been established should have a direct relationship with the clearance, and it should be possible to relate the slope of this line to the clearance. The determination of the slope of this line is the basis for the method here proposed for the estimation of glomerular filtration rate.

The method is applied as follows: a blood sample is obtained which serves to correct subsequent blood inulin analyses for the non-inulin chromogenic material. 0.15 g of inulin per kilo body weight is injected intravenously. At approximately 2 and 3 hours after the completion of the injection blood samples are drawn. The exact times of the injection and the drawing of the second and third samples are noted. The inulin content of the 2 samples of blood is determined. On semi-logarithm paper the 2 blood samples are plotted on the logarithmic coördinate against time on the linear coördinate. A straight line is drawn between these 2 points, and the slope of the line determined by the formula:

$$\text{slope} = \frac{K - \log C}{t}$$

where K is a constant whose value is determined by extending the line to zero time and taking the log of the concentration at this point; C is the plasma inulin concentration in mg % at any given time, t, in minutes, after the inulin injection.

The relation of the slopes of the lines obtained above to inulin clearance as actually determined is shown in Fig. 1. The dots rep-

⁴ Smith, H. W., *The Physiology of the Kidney*, Oxford University Press, New York, 1937.

resent data on children ranging in age from 5 to 15 years. The inulin clearances corrected for body size were done according to the technic described by Alving and Miller⁴ and the inulin determinations by the method of Alving, Rubin, and Miller.⁵ Each point represents the average of 2 consecutive periods. The open circles represent points calculated from the data of Alving and Miller.⁴ A fairly good relationship is shown to exist between the 2 factors.

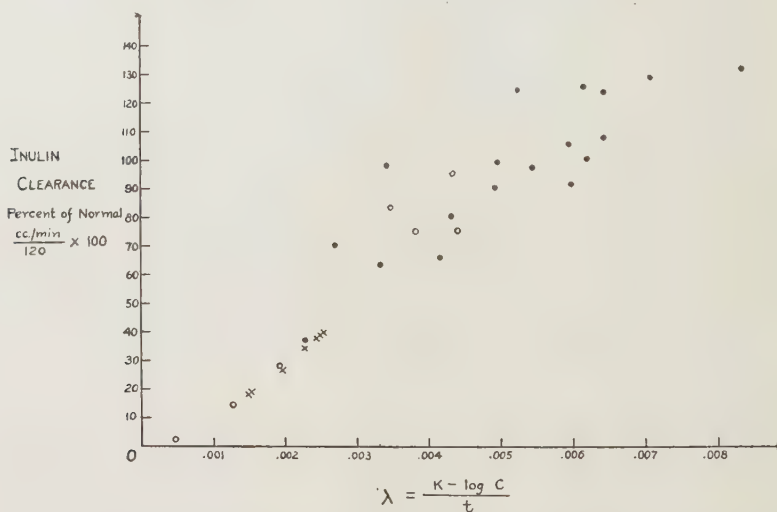


FIG. 1.

Relation of inulin clearances to slopes of lines representing fall in plasma inulin concentration following intravenous injection of inulin. Dots represent data on children from 5 to 15 years. Circles represent points calculated from the data of Alving and Miller. For explanation of crosses see text.

The slope of the line obtained as described appears to offer a fair estimation of the rate of glomerular filtration. The method would seem to be of value in instances in which the collection of urine specimens is very difficult and in which the error in collecting urines is probably greater than the errors inherent in the method. This would apply particularly to very young infants.

The application of this method to 7 apparently normal full-term newborns ranging in age from 4 to 9 days revealed slopes corresponding to inulin clearances ranging from 20 to 40% of normal (120 cc per minute). The slopes of these lines are plotted on Fig. 1 as crosses along the general line of the graph. The lines are shown as Group I in Fig. 2. That a straight line relationship in the decrease of the plasma inulin concentration, plotted logarithmically against time, does exist in newborns after an intravenous injection

⁵ Alving, A. S., Rubin, J., and Miller, B. F., *J. Biol. Chem.*, 1939, **127**, 609.

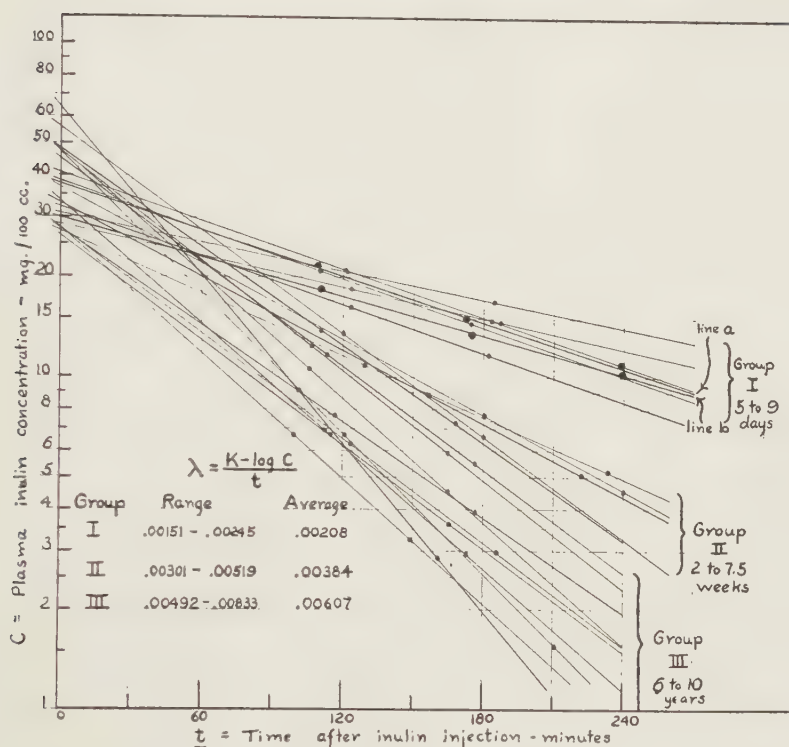


FIG. 2.

Lines obtained in different age groups by plotting plasma inulin concentrations logarithmically against time following intravenous injection of inulin.

of inulin is shown in Fig. 2 in which 3 points on the line were obtained in 2 instances (lines *a* and *b*). The lines obtained in 4 older infants ranging in age from 14 days to 7½ weeks and apparently without kidney disease (Group II) and the lines obtained by repeated determinations on 2 children 6 and 10 years of age (Group III) are also plotted on Chart 2. The slopes of the lines obtained on Group II correspond to clearances ranging from 50 to 90% of normal, and the slopes of the lines of Group III correspond to normal clearances. These results suggest a definitely diminished inulin clearance in newborns, which rapidly disappears during early infancy, perhaps in some cases as early as the 14th day. These results correlate with the histological characteristics of the renal glomerulus in early postnatal life, a recent study of which is reported by Gruenwald and Popper,⁶ who showed that in embryonic life there exists a resistance against filtration due to a matting together of the glomerular loops which are invaginated in a sac of high columnar epithelium.

⁶ Gruenwald, P., and Popper, H., *J. Urol.*, 1940, **43**, 452.

In early postnatal life the peaks of the loops are still covered by this type of epithelium, while in the second year the histological appearance is similar to that of the adults.

Further studies are needed to prove the validity of this observation and to further elucidate its mechanism.

11560

Chromodacryorrhea, a New Criterion for Biological Assay of Acetylcholine.

SHIRO TASHIRO, CARL C. SMITH,* ELIZABETH BADGER AND
EDWARD KEZUR.

From the Department of Biochemistry, College of Medicine, The University of Cincinnati.

The peculiar phenomenon of the shedding of bloody tears by rats was reported in connection with the studies of dacryorrhetin, a compound prepared from muscle.^{1, 2} This phenomenon is so unique and easily distinguishable from ordinary lacrimation that the term, chromodacryorrhea, is proposed for it. It has served as a very convenient criterion for the biological assay of dacryorrhetin.

When Selye³ published a paper in which he quotes Freud's observation⁴ that acetylcholine causes rats to shed tears tinged red by blood,† one of us (T) examined chromodacryorrhetic and other properties of acetylcholine to see if dacryorrhetin could be in reality acetylcholine.⁵ The results of these investigations showed that these two compounds are not identical and at the same time suggested a possibility of using chromodacryorrhea as a new criterion for a biological assay of acetylcholine. We have thus determined how small amounts of acetylcholine can be detected accurately by this criterion under different conditions.

* Charles Arnold Iglaue Fellow in Biochemistry.

1 Tashiro, Shiro, and Stix, Helen, *Biol. Bull.*, 1935, **64**, 327.

2 Tashiro, Shiro, *Proc. Am. Soc. Biochem.*, 1937, **8**, xeviii.

3 Selye, Hans, *Canadian Med. Assn. J.*, 1937, **36**, 200.

4 Freud, J., *Acta Brevia Neerl.*, 1933, **3**, 159.

† Although no casual observer would question the presence of blood in tears, and it gives a positive benzidine reaction and its bands are much like those of oxyhemoglobin when examined with a hand spectroscope, yet Tashiro and Badger have evidence that the red pigment in the bloody tears is not oxyhemoglobin.

5 Tashiro, Shiro, Kongressbericht, II. des XVI Internat. Physiologenkongress 1938, 46.

Experimental. The Criterion. The phenomenon of bloody tear flow in rats occurs within 2-5 minutes after an intraperitoneal injection, usually following salivation and clear tears; and it is almost instantaneous with an intravenous injection of effective doses. If salivation and clear tears are absent within 2-5 minutes after injection, usually bloody tears never flow. In the borderline cases, the naked eye may fail to recognize bloodiness in the tear, but with filter paper a slight tinge of red may be detected. In such a case, chromodacryorrhea is considered to be \pm .

Animals. For intraperitoneal injections, rats of any size, sex and age are used; but for intravenous injections, young rats weighing 100-200 g are chosen. For intravenous injection, the tail is carefully washed, immersed in warm water and immediately the solution is injected into the caudal vein by means of a 27 gauge needle with a tuberculin syringe.† The same rats can be used repeatedly if they are injected only once a day.

Eserine Treatment. Preliminary experiments on optimum conditions for eserization of rats show that an intraperitoneal injection of 50 γ eserine sulfate per 100 g body weight is best for both intraperitoneal and intravenous injections of acetylcholine. In either case, an acetylcholine solution is injected after the eserine effect becomes obvious (5-10 minutes). A convenient solution for eserization is prepared by dissolving 10 mg of eserine sulfate in 20 cc H_2O , 0.1 cc of which contains 50 γ , the exact quantity necessary for each 100 g body weight.

The Minimum Chromodacryorrhetic Doses. The chromodacryorrhetic response was determined for 4 different cases, intraperitoneal injection with and without eserine and intravenous injection with and without eserine. The minimum effective doses chosen are those amounts with which a majority of rats shed bloody tears from both eyes detectable with the naked eyes without the aid of filter paper. They are expressed as the weights of acetylcholine iodide on the basis of 100 g body weight of the rats. The results of these experiments are given in Table I, in which the value for intraperitoneal dosage without eserine represents an average, as one naturally expects a wider variation under this condition. Freud states the normal effective dose (intraperitoneal) in rats of 100-120 g to be ± 6 mg, which we presume to be of the iodide and to mean that the minimal dosages are around 6 mg.

The fact that the rat is more sensitive to intravenous injection

† Thanks are due to Dr. A. R. Sabin for his kind suggestions as to technic of intravenous injection in rats.

TABLE I.
Minimal Chromodacryorrhetic Dosages of Acetylcholine Iodide, Calculated on the
Basis of 100 g Body Weight of Rats.

Mode of injection	Without eserine	With eserinizied rats
	γ	γ
Intraperitoneal	2,000	50.0
Intravenous	10-15	0.2

without eserine than to the intraperitoneal with eserine, and the fact that the eseriniziation increases sensitivity only about 50 times (10-15 γ to 0.2 γ) in the case of intravenous injection are rather interesting in view of the fact that one of us (S)⁶ found an exceedingly small amount of the esterase in the serum of rats as compared to human serum.

The Quantitative Assay. These minimum effective dosages can be used for the basis of the quantitative assay of acetylcholine, if less than 1 cc of the solution contains more than 0.2 γ . Without eseriniziation, the intravenous injection gives fairly uniform results, but the results with intraperitoneal injection without eserine should be considered preliminary and approximate. The latter will be found useful when the available sample is large or highly concentrated such as in the study of acetylcholine synthesis and it will be exceedingly accurate for the estimation of other choline derivatives in which chromodacryorrhea is not enhanced by eseriniziation.

In either event, the quantitative assay of an unknown is done by determining the minimum positive and the maximum negative doses, accuracy depending upon the range between these two doses. For the actual analysis, a number of rats are weighed and about five rats are eserinizied at the same time. Usually 0.1 cc of the solution per 100 g body weight is injected intraperitoneally. If the reaction is positive, a smaller amount or a diluted solution is similarly injected. If negative, the maximum injectable amount of the original solution should be injected intravenously to see if a measurable amount of acetylcholine is present. If positive a smaller amount or a diluted solution is injected. The cc per 100 g body weight with which the majority of rats give the reaction contain the minimum effective amount. Thus the concentration per cc of the original unknown solution will be: for intraperitoneal injection, $50 \gamma \div \text{cc per 100 g body weight containing the minimum effective dose}$; and for intravenous injection, $0.2 \gamma \div \text{cc per 100 g body weight}$. If the original solution is diluted, the value, of course, should be multiplied by the factor.

⁶ Smith, Carl C., unpublished data.

Individual Variation of Rats. One should remember that occasionally he will find a rat which does not respond to dosages several times as large as the minimal effective dose. These variations must be due to the abnormal condition of Harder's glands, as one occasionally finds one or both glands lacking in some rats. There will be also a slight variation around the minimal dosage. Thus, if a large number of eserinizated rats are injected intravenously with 0.2 γ acetylcholine iodide, there will be some which fail to shed bloody tears, although usually all will shed them with 0.3 γ dose. This variation is not due to a difference in sex or age. It is not probably due to variation in the esterase activity since a similar variation does occur when examined with the minimal dose of another choline derivative which is not destroyed by esterase. It is most likely due to a physiological condition of the glands, in which a slight change might be sufficient to produce a different response to the minimal dose. In any event, by selecting the minimum dosages in which the majority of rats shed bloody tears, this variation can be ignored.

Specificity of This Test. Chromodacryorrhea is not a specific test for acetylcholine any more than other methods of biological assay of acetylcholine. According to Freud, 0.5-1 mg of pilocarpine gives the reaction, but 20-80 mg of choline have no effect. We also find that choline and eserine do not produce chromodacryorrhea, although we noticed a few cases in which the animals shed the bloody tears during their death struggle following injection of very high toxic dosages of these compounds. Such rare cases will not interfere with the assay, for a mere dilution will eliminate the reaction. A powerful chromodacryorrhetic action of dacryorrhetin, although prepared from muscle, should not interfere with this test in a tissue analysis for acetylcholine, as this substance exists in the body as prodacryorrhetin which has no chromodacryorrhetic action. If necessary, one can easily distinguish dacryorrhetin from acetylcholine by comparing the effective doses with and without eserine, as the action of the former will not be appreciably enhanced by eserine.

Summary. 1. By using chromodacryorrhea, the phenomenon of the shedding of bloody tears by rats, as a criterion, acetylcholine can be detected in as small amount as 0.2 γ . 2. Since there are 4 ranges of minimal dosages detectable with this phenomenon, from 2 mg to 0.2 γ , depending on the mode of injection and treatment, this same criterion can be used for a wide range of concentration of acetylcholine with accuracy.

Effect of Depriving Newborn of Placental Blood upon Early Postnatal Blood Picture.

Q. B. DEMARSH, W. F. WINDLE* AND H. L. ALT.†

From the Anatomical Laboratories, Northwestern University Medical School, Chicago.

Within the last few years, the collection of postpartum placental blood for "blood banks" has been strongly advocated not only in Russia¹ but also in Canada² and in this country.^{3, 4, 5} It has been pointed out that this is an inexhaustible and "lucrative" source of blood, satisfactory for transfusion purposes, and that its collection has no deleterious effect upon the mother. Possible effects upon the child have been disregarded because it has already become a rather common obstetrical practice to clamp the umbilical cord promptly at birth in spite of the fact that most of the placental blood normally drains into the body of the infant within a few minutes when the umbilical cord is not clamped immediately after delivery.⁶ When the cord is clamped immediately, the infant is deprived of an alarming proportion of its total blood volume and usable iron at the very beginning of extra-uterine life. We have obtained proof that this blood-letting at birth affects the blood picture of the newborn significantly. The practice should be strongly condemned.

We have determined the amount of hemoglobin and number of red blood corpuscles in blood taken from the mother on the day of birth, in cord blood at birth, in blood from the newborn (heel) 15 to 75 minutes after birth, and from the infant at one, 3 to 4, and 6 to 7 days after birth in 2 series of patients. In one series of 25, the umbilical cord was clamped within 30 seconds after delivery; in another series of 29, clamping was delayed until pulsations had

* Aided by a grant from the John and Mary R. Markle Foundation.

† Department of Medicine. This investigation was conducted at the Cook County Hospital, on the service of Dr. David S. Hillis, whose cooperation is greatly appreciated by the authors.

¹ Bruskin, Y. M., and Fackerova, P. S., *Soviet Vrach. Zhur.*, 1936, No. 20, p. 1546 (cited by Gwynn and Alsever).

² Goodall, J. R., Anderson, L. O., Altimas, G. T., and McPhail, F. L., *Surg., Gyn. and Obst.*, 1938, **66**, 176.

³ Grodberg, B. C., and Carey, E. L., *New Eng. J. Med.*, 1938, **219**, 471.

⁴ Gwynn, C. A., and Alsever, J. B., *Am. J. Med. Sc.*, 1939, **198**, 634.

⁵ Heyl, W. M., *Am. J. Obst. and Gyn.*, 1940, **39**, 679.

⁶ Haselhorst, G., and Allmeling, A., *Z. f. Geburtsh. u. Gynäk.*, 1930, **98**, 103.

ceased and the placenta had separated. Hemoglobin was determined in grams percent with a calibrated Hellige-Sahli instrument. Red blood corpuscle counts were made with a Spencer hemocytometer (N.B.S.). Reticulocyte counts were obtained in 4 infants whose cords had been clamped immediately and in 4 in which clamping had been delayed.

Complete data will be published in a future article. The umbilical cord blood at the end of gestation in both series contained about 15.7 g of hemoglobin per 100 cc and about 4.5 millions of red corpuscles per cmm. Within a brief period of time after birth—averaging less than an hour in our experiments—the amount of hemoglobin and number of corpuscles in the newborn's blood increased markedly. Values rose to 21.3 g % Hb and 5.93 millions R.B.C. in those infants allowed to retrieve their placental blood. In those deprived of the placental blood by immediate clamping of the umbilical cord, the values rose only to 18.9 g % and 5.57 millions R.B.C. in the same interval. The amount of hemoglobin and number of corpuscles increased further during the course of the first postnatal day (22.5 g % Hb and 6.22 million R.B.C.) in the infants receiving their normal share of placental blood, but not in those deprived of it. The former group maintained higher values throughout the period under investigation.

One difference between the two experimental groups manifested itself on about the fourth day of life. Hemoglobin reached a peak in both series at that time, but those infants deprived of placental blood at birth exhibited an increase in hemoglobin, amounting to 1.4 g % above the one day average; while the other group showed a rise of only 0.5 g %. In neither series did the red corpuscle count rise.

Reticulocytes reached a peak at one day after birth. In a group of 4 infants whose cords were clamped immediately, this amounted to 8.3 % as compared with 4.7 % in the 4 infants of the other group.

Our experiments have demonstrated that failure to allow the placental blood to return in large measure to the infant at the time of delivery is equivalent to submitting the newborn to a hemorrhage. Acceleration of hemopoiesis appears to occur in an attempt to make up this loss. It can not be doubted that a drain is placed upon the infant's iron reserve, and at this time in life it can ill afford such a loss, for it must get along with what iron it has in its body at birth until the nursing period is passed.

Our experiments may help to reconcile a persistent disagreement in the literature regarding hemoglobin and corpuscular values in

man at birth.⁷ Some investigators have obtained results comparable with our determinations in cord blood and others have found higher values similar to ours in blood drawn from the infant after delivery. The exact source and time of collection of newborn blood have not always been stated. The difference which we have found between cord (venous) blood at the moment of birth and capillary blood from the infant less than an hour later is truly surprising but may be more apparent than real. A similar difference between venous and capillary blood has been reported in pernicious anemia but not in normal adults.⁸ It is possible that macrocytes in the blood of infants as well as of P.A. patients block some of the capillaries and thus effect a concentration of corpuscles.

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Cerebellar Action Potentials in Response to Stimulation of Cerebral Cortex.

HOWARD J. CURTIS. (Introduced by E. K. Marshall, Jr.)

From the Department of Physiology, The Johns Hopkins University School of Medicine, Baltimore, Md.

The problem of functional localization in the cerebellum is one which has received considerable attention. Recent comparative anatomical studies¹ and ablation experiments² have supported a division of the cerebellum based on afferent fiber connections. Recently Dow³ has recorded action potentials in the cerebellum as a result of stimulating various afferent fiber tracts, and his results are in accord with Larsell's anatomical findings. The present work is an attempt to explore by the oscillographic method the projections of the cerebral cortex to the cerebellar cortex.

Methods and Results. Twelve cats, under barbiturate anesthesia, were used in this work. The method of stimulating and recording is described elsewhere.⁴ Single electrical shocks were

⁷ Waugh, T. R., Merchang, F. T., and Maughan, G. B., *Am. J. Med. Sc.*, 1939, **198**, 646.

⁸ Duke, W. W., and Stoffer, D. D., *Arch. Int. Med.*, 1922, **30**, 94.

¹ Larsell, O., *Arch. Neurol. Psychiat.*, 1937, **38**, 580.

² Fulton, J. F., and Dow, R. S., *Yale J. Biol. Med.*, 1937, **10**, 89.

³ Dow, R. S., *J. Neurophysiol.*, 1939, **2**, 543.

⁴ Curtis, H. J., 1940, in preparation.

applied to the cerebral cortex by means of bipolar electrodes about 1 mm apart resting lightly on the pia. Monopolar recording was employed, the active electrode being a chlorided silver wire in light contact with the pia of the cerebellar cortex. Fig. 1 is a record of a cerebellar action potential obtained in this way. Following the shock artefact there is a surface positive wave having a latency of about 25 msec to the crest of the wave, and a second positive wave having a latency as long as 200 msec. The second wave by no means always accompanies the first, but the factors causing this wave have not been determined.

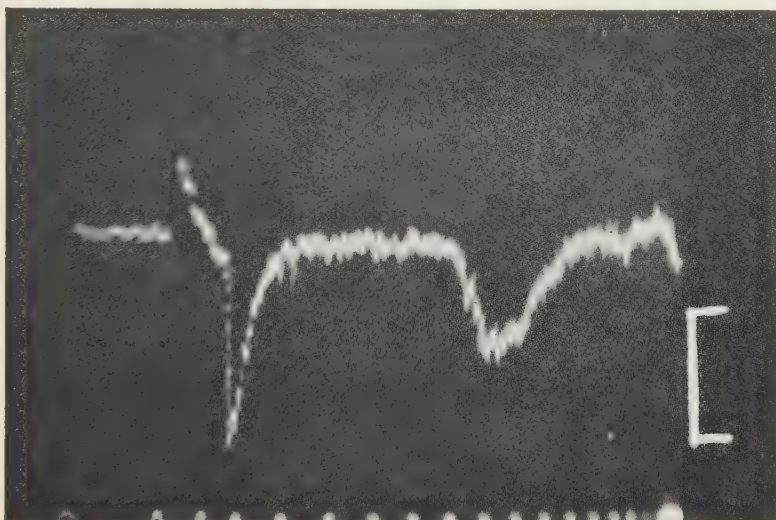


FIG. 1.

Cerebellar action potential recorded from Crus I, Lobulus ansiformis, as a result of a single electrical shock applied to the middle suprasylvian gyrus. The initial upward deflection is the shock artefact. Downward deflection indicates a surface positive potential. Time marks, 60 cycle; calibration mark, 200 μ V.

Stimulation of one cerebral cortical point may produce simultaneous potentials in as many as 14 distinct points on the cerebellar cortex. Considering the extensive foliation of this structure, it must be true that only a small fraction of the total surface was explored; the total number of points which yield potentials must be far greater than this. The most easily detected potentials are on the contralateral side, and in general for each of these potentials there is a smaller potential on the ipsilateral side at a point roughly symmetrical to it.

If the stimulus is well localized the cerebellar potentials may be very sharply localized; a point exhibiting a large potential may be

only 1 mm away from a point which shows no measurable potential. On the other hand, when the stimulating electrodes are moved the pattern of the potentials on the cerebellar cortex is changed, but the potential at any single point may remain almost unchanged while the stimulating electrodes are moved by as much as 2 cm. This phenomenon is not due to a spread of the stimulating current, since a displacement of the stimulating electrodes by only 1 mm will often very markedly change the pattern of the cerebellar response.

None of the areas in the cerebral cortex which have been explored has failed to produce at least one potential in the cerebellum, and these areas include the sigmoid gyrus, marginal gyrus, middle suprasylvian gyrus, and middle ectosylvian gyrus. It was found that with the exception of the declive and tuber vermis all of these areas project to the neocerebellum¹ and to the posterior part of the anterior lobe. Fig. 2 shows a dorsal view of the cerebellum; the cross hatching indicates regions from which potentials have been recorded, shading indicates regions explored without finding potentials, and the unshaded regions were not explored. There seem to be no anatomically distinct regions which can be said to be associated with particular areas of the cortex. In other words, any given region in the cross-hatched areas of Fig. 2 may receive im-

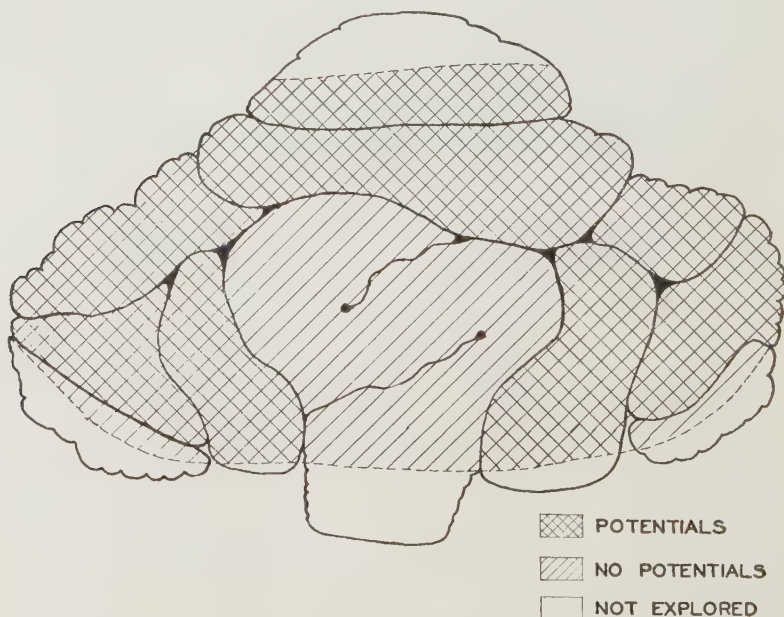


FIG. 2.

Diagram of dorsal view of cat's cerebellum showing regions from which potentials have been obtained as a result of stimulating the cerebral cortex.

pulses from any or all of the areas of the cerebral cortex which were explored.

The application of a small quantity of 0.3% picrotoxin solution to the surface of the pia over a small region exhibiting one of these potentials, radically changed the size and shape of the recorded potential. The initial surface positive wave was immediately followed by a large surface negative wave, giving the response a di-phasic appearance. The effect seems to be quite similar to that observed in the cerebral cortex⁴ except that it is necessary to use more concentrated solutions in the case of the cerebellum to evoke the response, a fact which may explain why Dow⁵ failed to observe "strychnine spikes" in the cerebellum.

Discussion and Conclusions. These results appear to support and extend the ideas of functional localization in the cerebellum⁶ as opposed to those of anatomical localization.⁷ They indicate that there is no region in the neocerebellar cortex which can be said to be particularly related to any region of the cerebral cortex. Since the cerebellum has come to be known as an organ of synthesis and co-ordination, it would hardly seem strange (a) that many different functional areas in the cerebral cortex are connected with a single point in the cerebellum, and (b) that a single cerebral cortical point projects to a number of cerebellar foci. This is in good agreement with Dow's work³ in which he found that stimulation of spinal nerves produced a pattern of potentials in the anterior lobe of the cerebellum which changed very little when nerves from different parts of the body were stimulated.

The fact that most of the individual cerebellar potentials obtained in the present study are very sharply localized perhaps throws some doubt on the concept of mass function of the cerebellum, at least as far as the afferent connections are concerned. The results indicate that a relatively small number of cerebral cortical efferents are capable of exciting a large number of small isolated cerebellar units. Thus it appears probable that a single cortical efferent makes synaptic connections with several cerebellar afferents in the pontine nuclei. The occurrence of multiple potentials over the surface of the cerebellar cortex cannot be due to spread of excitation there, since there is no very appreciable difference in latency between the potentials recorded from the different points which form any one pattern.

The distribution of potentials shown in the map of Fig. 2 is, in

⁵ Dow, R. S., *J. Physiol.*, 1938, **94**, 67.

⁶ Sherington, C. S., in Schäfer, *Textbook of Physiology*, 1900, **2**, 884.

⁷ Bolk, L., *Das Cerebellum der Saugetiere*, 1906, Harlem, Bohn.

general, what one would expect from the known cerebellar afferent connections assuming that all cerebro-cerebellar connections are effected by synapses in the pontine nuclei. Dow,³ by directly stimulating the pons, obtained potentials not only in all areas from which potentials were recorded in the present work, but also in the declive and tuber vermis, pyramis, and paraflocculus. The fact that no potentials have been obtained from the declive and tuber vermis on cerebral cortical stimulation is interesting in view of the fact that Larsell¹ includes this part of the organ in the neocerebellum. It should be emphasized, however, that one must interpret the absence of potentials with extreme caution.

SECRETARY'S REPORT

April 1, 1939—March 31, 1940

The annual meeting of the Council took place March 15, 1940 at New Orleans. Members present: Doctors Doisy, Gasser, Leake, Meek, Smith, Soskin and Thienes; by proxy, Doctor Adolph (for Dr. Hodge), Dr. Green (for Dr. Ingram), Dr. Myers (for Dr. Hayman), Dr. Roe (for Dr. Leese), and Dr. Tainter (for Dr. Meyer).

Membership. The Council elected 103 applicants for membership, previously approved unanimously by the National Membership Committee. The Council elected 5 applicants not unanimously approved by the Membership Committee. The Council approved the recommendation of the National Membership Committee that action on 4 applications be deferred pending further publication. (The National Membership Committee by a majority voted to defer action on 23 applications pending further publication.)

The Council unanimously voted that only those applications for membership received by the Secretary-Treasurer by December 15 shall be forwarded to the National Membership Committee for report to the Council at its annual meeting.

The following resignations were accepted with regret: Doctors M. L. Anson, I. S. Falk, F. H. Falls, F. B. Flinn, H. J. Fry, R. Guy, W. W. Hamburger, J. Markowitz, F. L. Meleney, L. F. Rettger, H. B. Richardson, H. Sommer, A. Steindler and W. G. Young.

Five members were declared in arrears and dropped from the membership list.

Two members in China were given an extension of time to clear their accounts.

Doctors Gary N. Calkins and P. A. Levene were declared emeritus members.

Ballot. The Council unanimously voted that the ballot with nominees for national offices shall be distributed to members within 30 days after the annual meeting of the Council.

Honorary Members. The Council approved the recommendation of the Committee that the Society discontinue the election of honorary members.

Finances. The Treasurer reported a deficit of \$2,319 for the 11 months' period April 1, 1939 to March 1, 1940, or about \$2,500 for the fiscal year. (Details are given in the Treasurer's annual report). This deficit was due primarily to the following:

1. Greater increase in size of PROCEEDINGS than had been anticipated.
2. The low charge for excess space, 20 percent of cost.
3. The low membership dues.

This deficit will be paid from accumulated profits of preceding years, viz., Surplus Fund.

To meet the expected deficit for the coming year, the Council approved the following changes:

1. Increase the charge for excess space from 20 to 40 percent of cost.
2. Increase subscription price (to non-members) from \$6.00 to \$7.50 a year.
3. Use interest of Surplus Fund.

By-Laws. Suggested changes in the by-laws were proposed. It was decided to request the President to appoint a committee of three in or near New York to consult with the Secretary-Treasurer to propose changes in the by-laws.

Editors. The following were elected to the Editorial Board:

C. A. Elvehjem, in biochemistry of vitamins

R. J. Dubos, in physiology and metabolism of bacteria

E. M. K. Geiling, in pharmacology

E. M. Marshall, Jr., in pharmacology.

National Membership Committee. This committee held its annual meeting March 15, 1940, to reconsider those applications for membership which had not been unanimously approved by them. Their findings were reported to the Council, (see above). A further effort was made to define more precisely what constitutes eligibility to membership.

PAST OFFICERS

<i>Date</i>	<i>President</i>	<i>Vice-President</i>	<i>Secretary</i>	<i>Treasurer</i>
1903-04	S. J. Meltzer	W. H. Park	W. J. Gies	G. N. Calkins
1904-05	S. J. Meltzer	J. Ewing	" "	" "
1905-06	E. B. Wilson	E. K. Dunham	" "	" "
1906-07	S. Flexner	E. K. Dunham	" "	" "
1907-08	S. Flexner	T. H. Morgan	" "	" "
1908-09	F. S. Lee	T. H. Morgan	" "	G. Lusk
1909-10	F. S. Lee	W. J. Gies	E. L. Opie	" "
1910-11	T. H. Morgan	W. J. Gies	" "	" "
1911-12	T. H. Morgan	P. A. Levene	G. B. Wallace	" "
1912-13	J. Ewing	P. A. Levene	" "	C. Norris
1913-14	J. Ewing	C. W. Field	H. C. Jackson	" "
1914-15	G. Lusk	W. J. Gies	" "	J. R. Murlin
1915-16	G. Lusk	G. N. Calkins	" "	H. C. Jackson
1916-17	J. Loeb	W. J. Gies	" "	" "
1917-19	W. J. Gies	J. Auer	" "	" "
1919-21	G. N. Calkins	G. B. Wallace	" "	" "
1921-23	G. B. Wallace	J. W. Jobling	" "	" "
1923-24	H. C. Jackson	J. W. Jobling	" "	V. C. Myers
1924-25	H. C. Jackson	J. W. Jobling	" "	A. J. Goldforb
1925-27	J. W. Jobling	S. R. Benedict	" "	" "
1927-29	S. R. Benedict	P. Rous	" "	" "
1929-30	P. Rous	D. Marine	" "	" "
1930-31	P. Rous	D. J. Edwards	" "	" "
1931-32	D. J. Edwards	A. R. Dochez	" "	" "
1932-34	A. R. Dochez	E. L. Opie	" "	" "
1934-36	E. L. Opie	P. E. Smith	" "	" "
1936-37	P. E. Smith	E. F. DuBois	" "	" "
1937-39	H. S. Gasser	J. T. Wearn	" "	" "
1939-40	J. T. Wearn	C. D. Leake	" "	" "

SECTIONAL MEETINGS AND MEMBERSHIP

Cleveland, Ohio

Chairman: H. Feil. Secretary: W. Hambourger. Members: 42.

Meetings: Western Reserve University, October 13, 1939

November 10, 1939

December 8, 1939

January 12, 1940

February 9, 1940

March 8, 1940

April 12, 1940

District of Columbia

Chairman: C. E. Leese. Secretary: D. B. Jones. Members: 42.
Meetings: U. S. Public Health Service, December 7, 1939

Illinois

Chairman: T. E. Boyd. Secretary: A. Weil. Members: 139
Meetings: University of Chicago October 24 1939
University of Illinois Medical School, December 12, 1939
Northwestern University, January 23, 1940
University of Chicago, April 9, 1940
Northwestern University, May 21, 1940

Iowa

Chairman: E. D. Plass. Secretary: T. L. Jahn. Members: 39
Meetings: State University of Iowa, February 14, 1940
April 26, 1940

Minnesota

Chairman: F. H. Scott. Secretary: F. H. Scott. Members: 51
Meetings: University of Minnesota, October 18, 1939
December 20, 1939
February 21, 1940
March 20, 1940
April 17, 1940
May 15, 1940

Missouri

Chairman: A. B. Hertzman. Secretary: H. L. White. Members: 55
Meetings: St. Louis University Medical School, October 11, 1939
Washington University Medical School, December 13, 1939
St. Louis University Medical School, February 14, 1940
Washington University Medical School, April 10, 1940
St. Louis University Medical School, May 8, 1940

New York

Chairman: J. C. Hinsey. Secretary: I. Greenwald. Members: 453
Meetings: New York Academy of Medicine, October 18, 1939
New York Medical College, November 22, 1939
Cornell University Medical College, January 24, 1940
New York University, February 28, 1940
Rockefeller Institute, April 24, 1940
College of Physicians and Surgeons, May 22, 1940

Pacific Coast

Chairman: A. W. Meyer. Secretary: C. Weiss. Members: 101
Meetings: University of California, October 21, 1939
Stanford University Medical School, December 8, 1939
University of California Hospital, February 7, 1940
Mount Zion Hospital, March 8, 1940

Peiping, China

Chairman: A. B. Fortuyn. Secretary: F. T. Chu. Members: 30

Meetings: Peiping Union Medical College, October 25, 1939

February 7, 1940

May 9, 1940

Southern

Chairman: H. S. Mayerson. Secretary: R. Ashman. Members: 41

Meetings: Tulane University, November 3, 1939

January 26, 1940

Louisiana State University, May 3, 1940

Southern California

Chairman: E. Bogen. Secretary: M. S. Dunn. Members: 40

Meetings: University of Southern California, October 12, 1939

Los Angeles County General Hospital, November 30, 1940

Monterey, Calif., December 20, 1940

Scripps Institution of Oceanography, March 2, 1940

University of California, Los Angeles, May 2, 1940

Western New York

Chairman: E. F. Adolph. Secretary: H. C. Hodge. Members: 62

Meetings: Syracuse University, October 14, 1939

University of Buffalo, December 9, 1939

University of Rochester, February 17, 1940

Cornell University, May 18, 1940

Wisconsin

Chairman: W. E. Sullivan. Secretary: O. O. Meyer. Members: 41

MEMBERSHIP

Members, March 31, 1939.....	1488	
Electd during year.....	23	
Total		1511
Resignations	25	
Deaths	11	
Arrears	10	46
Total Membership, March 31, 1940.....		1465
	1930	1940
Membership	1026	1465
Subscriptions, March 31, 1940.....		608

DEATHS OF MEMBERS

The Council records with regret the deaths of the following members: Doctors H. V. Atkinson, A. H. W. Caulfeild, C. B. Coulter, D. M. Cowie, G. E. Cullen, H. W. Cushing, W. T. Dawson, F. P. Gay, R. H. Jaffe, F. S. Lee, H. A. McCordock, E. B. McKinley, D. Perla, O. H. Plant, and I. C. Wen.

Dr. O. H. Plant was a member of the Editorial Board from 1936 to the fall of 1939. With his death the Society lost a genial, devoted, considerate and eminently fair member of the Board. He aided materially in developing an ever better standard for the PROCEEDINGS.

TREASURER'S REPORT

April 1, 1939-April 1, 1940

Balance on hand, April 1, 1939..... \$ 7,433.16

Income

Dues	\$ 5,959.25
Reprints	4,120.48
Space	1,244.45
Cuts	789.12
Changes	66.39
Subscriptions	3,814.48
Back Numbers	185.54
Interest from special accounts.....	77.64
Miscellaneous	25.62
	<hr/>
	\$16,282.97

Total Cash Available, April 1, 1939-April 1, 1940..... \$23,716.13

Disbursements

Printing	\$10,958.70
Reprints	4,053.38
Cuts	836.58
	<hr/>
	\$15,848.66

Office Supplies, Postage, Telephone.....	686.45
Salary	2,205.00
Storage and Insurance	71.13
Miscellaneous	148.47
	<hr/>
	\$ 3,111.05

	\$18,959.71
Cash balance, April 1, 1940.....	4,756.42
	<hr/>
	\$23,716.13

SUMMARY

Income (net)	\$16,282.97
Disbursements (net)	18,959.71
	<hr/>

Deficit \$ 2,676.74

Interest from Surplus and Endowment Funds to be used	1,161.51
	<hr/>

Net Deficit \$ 1,515.23

Bills receivable—\$889.98

Bills payable—None.

TREASURER'S REPORT

FUNDS

Endowment Fund

April 1, 1939.....	\$17,304.68	
Interest to April 1, 1940.....	661.27	
		\$17,965.95
Invested in New York Title and Mortgage Co.....	\$ 5,880.00	
Title Guarantee and Trust Co.....	2,000.00	
Lawyers Mortgage Co.....	1,500.00	
Bowery Savings Bank.....	2,989.46	
United States Savings Bonds.....	1,875.00	
Industrial Bonds	3,721.49	
		\$17,965.95

Surplus Fund

April 1, 1939	\$10,924.16	
Interest to April 1, 1940.....	500.24	
		\$11,424.40
Invested in Title Guarantee and Trust Co.....	\$ 2,850.50	
Harlem Savings Bank.....	824.71	
United States Savings Bonds.....	1,875.00	
Industrial Bonds	5,874.19	
		\$11,424.40

Life Membership Fund

Invested in Railroad Federal Savings and Loan.....	\$ 75.00
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Auditors' Report

We the undersigned have this day examined the Treasurer's report and find it to agree with the books of the Society. We believe that the records of the financial transactions are accurate and in good order.

We reiterate the request of previous committees that a certified public accountant should be engaged to make periodic audits of the Treasurer's records for the protection of the Secretary-Treasurer and the members of the Auditing Committee.

(Signed) ALEXANDER B. GUTMAN
HOMER W. SMITH
WILLIAM S. TILLET

May 3, 1940.

MEMBERS' LIST

HONORARY MEMBERS

Flexner, Simon.....	Rockefeller Inst.
Howell, William H.....	Johns Hopkins Univ.
Porter, William	Harvard Univ.
Richet, Charles.....	Paris, France
Von Muller, Friedrich.....	Munich, Germany

MEMBERS

A bramson, D. I.....	May Inst. Med. Research, Cincinnati
Abramson, H. A.....	Coll. Physicians and Surgeons, New York
Abt, Arthur F.....	Northwestern Univ.
Adams, A. Elizabeth.....	Mount Holyoke Coll.
Adams, William E.....	Univ. of Chicago
Addis, Thomas	Stanford Univ. Med.
Adlersberg, D.....	Beth Israel Hosp., N. Y.
Adolph, E. F.....	Univ. of Rochester Med.
Adolph, W. H.	Peiping, China
Alexander, Harry L.....	Washington Univ.
Allen, Bennet M.....	Univ. of Calif., L. A.
Allen, Edgar	Yale Univ.
Allen, William F.....	Univ. of Oregon
Alles, G. A.....	San Marino, Calif.
Almquist, H. J.....	Univ. of Calif.
Alt, Howard L.	Northwestern Univ. Med.
Althausen, T. L.....	Univ. of Calif. Med.
Altschule, M. D.....	Beth Israel Hosp., Boston
Altshuler, S. S.....	Wayne Univ. Med.
Alvarez, Walter C.	Mayo Clinic
Alving, A. S.....	Univ. of Chicago
Amberg, Samuel.....	Mayo Clinic
Amberson, W. R.....	Univ. of Maryland Med.
Amoss, Harold L.....	Rockefeller Inst.
Anderson, Dorothy H.....	Coll. of Phys. and Surg., N. Y.
Anderson, H. H.....	Peiping Union Med. Coll.
Anderson, John E.....	Univ. of Minn.
Anderson, John F.....	E. R. Squibb & Son
Anderson, Rudolph J.....	Yale Univ.
Anderson, William E.....	Springfield, Mass.
Andrews, Edmund	Bloomington, Ill.
Ansbacher, Stefan.....	Squibb Inst., New Brunswick, N. J.
Antopol, William.....	Beth Israel Hosp., Newark, N. J.
Apperly, Frank L.....	Med. Coll. of Va.
Armstrong, Charles.....	National Inst. of Health, Washington

Arnold, Lloyd.....	Univ. of Ill.
Aronson, J. D.....	Henry Phipps Inst.
Asdell, S. A.....	Cornell Univ.
Asher, Leon.....	Berne, Switzerland
Ashman, Richard.....	Louisiana State Univ.
Asmundson, V. S.....	Univ. of Calif.
Atchley, D. W.....	Presbyterian Hosp., N. Y. C.
Atwell, Wayne J.....	Univ. of Buffalo
Aub, Joseph C.....	Huntington Memorial Hosp., Boston
Auer, John.....	St. Louis Univ.
Austin, J. Harold.....	Univ. of Pa.
Avery, B. F.....	Am. Univ. of Beirut
Avery, O. T.	Rockefeller Inst., N. Y. C.
Avery, Roy C.....	Vanderbilt Univ.
Aycock, W. L.....	Harvard Med.

B abkin, Boris P.....	McGill Univ.
Bachem, Albert.....	Univ. of Ill. Med. Coll.
Baehr, George.....	Mt. Sinai Hosp., N. Y. C.
Bagg, Halsey J.....	Memorial Hosp., N. Y. C.
Bahrs, Alice M.....	Portland, Ore.
Bailey, Cameron V.....	N. Y. Post-Graduate Med.
Baitsell, George A.....	Yale Univ.
Baker, Lillian E.....	Rockefeller Inst.
Bakwin, Harry.....	N. Y. Univ. Med. Coll.
Baldwin, Francis M.....	Univ. of S. Calif.
Ball, G. H.....	Univ. of Calif., L. A.
Ball, H. A.....	San Diego, Calif.
Balls, A. K.....	U. S. Dept. of Agr.
Banting, Frederick G.....	Univ. of Toronto
Barach, Alvan L.....	Coll. of Phys. and Surgeons, N. Y.
Barber, W. Howard.....	New York Univ. Med.
Barbour, Henry G.....	Yale Univ.
Barer, Adelaide P.....	State Univ. of Iowa
Barlow, O. W.....	Rensselaer, N. Y.
Barnett, George D.....	Stanford Univ.
Barr, David P.....	Washington Univ.
Barron, E. S. G.....	Univ. of Chicago
Barth, L. G.....	Columbia Univ.
Bartley, S. H.....	Washington Univ.
Bass, Charles.....	Tulane Univ.
Bast, T. H.....	Univ. of Wisconsin
Bates, R. W.....	Carnegie Inst. of Washington
Bauer, J. H.....	Rockefeller Inst.
Bauman, Louis.....	Presbyterian Hosp., N. Y. C.
Baumann, E. J.....	Montefiore Hosp., N. Y. C.
Baumberger, J. Percy.....	Stanford Univ.
Bayne-Jones, S.....	Yale Univ.
Bazett, H. C.....	Univ. of Pa.
Bean, John W.....	Univ. of Mich.

Beard, H. H.	Louisiana State Univ.
Beard, J. W.	Duke Univ.
Beard, P. J.	Stanford Univ.
Beck, Claude S.	Western Reserve Univ.
Becker, E. R.	Iowa State Coll.
Beckman, Harry	Marquette Univ. Med.
Beckwith, T. D.	Univ. of Calif., L. A.
Behre, Jeannette A.	Cornell Univ. Med. Coll.
Belding, David L.	Boston Univ.
Bender, M. B.	Mt. Sinai Hosp., N. Y.
Bengston, Ida A.	National Inst. of Health, Washington
Berg, B. N.	Columbia Univ.
Berg, C. P.	State Univ. of Iowa
Berg, William N.	N. Y. City
Bergeim, Olaf	Univ. of Ill.
Bergmann, Max	Rockefeller Inst.
Bernhard, Adolph	Lenox Hill Hosp., N. Y. City
Bernthal, T. G.	Univ. of Mich.
Berry, George P.	Univ. of Rochester Med.
Beutner, R.	Hahnemann Med. Coll., Philadelphia
Bierman, W.	Mt. Sinai Hosp., N. Y.
Bieter, Raymond N.	Univ. of Minn.
Bills, C. E.	Mead, Johnson and Co., Evansville, Ind.
Bing, Franklin C.	Am. Med. Assn., Chicago
Binger, Carl A. L.	Rockefeller Inst., N. Y.
Birkhaug, Konrad E.	Geofysisk Inst., Bergen, Norway
Bishop, George H.	Webster Groves, Mo.
Bishop, Katharine S.	Univ. of Calif.
Blackfan, K. D.	Harvard Med.
Blair, John E.	Hosp. for Joint Diseases, N. Y.
Blake, F. G.	Yale Univ.
Blakeslee, Albert F.	Sta. for Exp. Evolution, Cold Spring Harbor, N. Y.
Blalock, Alfred	Vanderbilt Univ. Med.
Blatherwick, Norman R.	Metropolitan Life Insurance Co., N. Y. City
Blau, Nathan F.	Cornell Univ. Med. Coll.
Blinks, L. R.	Stanford Univ.
Bliss, Sidney	Tulane Univ.
Bloch, Robert G.	Univ. of Chicago
Block, Richard J.	N. Y. State Psychiatric Inst.
Bloom, William	Univ. of Chicago
Bloomfield, A. L.	Stanford Univ. Med.
Bloor, W. R.	Univ. of Rochester
Blount, R. F.	Univ. of Minn.
Blum, Harold F.	Washington, D. C.
Blumberg, Harold	Johns Hopkins Univ.
Blumgart, H. L.	Beth Israel Hosp., Boston
Boek, Joseph C.	Marquette Univ.
Bodansky, A.	Hosp. for Joint Diseases, N. Y.
Bodansky, Meyer	John Sealy Hosp., Galveston, Texas
Bodansky, Oscar	New York Univ.

Bodine, J. H.	State Univ. of Iowa
Bogen, Emil	Olive View, Calif.
Boissevain, Charles H.	Colorado Coll.
Bollman, Jesse L.	Mayo Clinic
Bonner, James	Calif. Inst. of Technology
Boor, Alden K.	Univ. of Chicago
Boothby, Walter M.	Kahler Hosp., Rochester, Minn.
Boots, Ralph H.	Presbyterian Hosp., N. Y.
Borsook, Henry	Calif. Inst. of Technology
Bowen, B. D.	Buffalo Gen. Hosp.
Boyd, Eldon M.	Queens Univ., Canada
Boyd, Theo. E.	Loyola Univ.
Boyden, E. A.	Univ. of Minn. Med.
Bozler, Emil	Ohio State Univ.
Bradford, William L.	Univ. of Rochester
Bradley, H. C.	Univ. of Wisconsin
Brand, Erwin	N. Y. State Psychiatric Inst.
Branham, Sara E.	National Inst. of Health, Washington
Brewer, George	Univ. of Pa.
Brewer, Robert K.	Syracuse Univ.
Briggs, A. P.	Univ. of Georgia
Bronfenbrenner, J.	Washington Univ.
Bronk, D. W.	Univ. of Pa.
Brooks, Clyde	Louisiana State Univ.
Brooks, Matilda M.	Univ. of Calif.
Brooks, S. C.	Univ. of Calif.
Broun, G. O.	St. Louis Univ. Med.
Brown, J. Howard	Johns Hopkins Univ.
Brown, John B.	Ohio State Univ.
Brown, L. A.	Transylvania Coll., Lexington, Ky.
Brown, Rachel	N. Y. State Dept. of Health
Brown, Wade H.	Rockefeller Inst., Princeton
Browne, J. S. L.	Royal Victoria Hosp., Montreal
Bruger, Maurice	N. Y. Post-Graduate Med.
Brunschwig, Alexander	Univ. of Chicago
Buchanan, A. R.	Univ. of Colo.
Buchanan, Robert E.	Iowa State Coll.
Buehbinder, W. C.	Michael Reese Hosp., Chicago
Buell, Mary V.	Johns Hopkins Univ.
Bulger, H. A.	Washington Univ.
Bullowa, J. G. M.	Harlem Hosp., N. Y.
Bunting, C. H.	Univ. of Wisconsin
Burch, George E.	Rockefeller Inst.
Burch, John C.	Vanderbilt Univ. Med.
Burdon, Kenneth L.	Louisiana State Univ.
Burky, Earl L.	John Hopkins Hosp.
Burns, E. L.	Louisiana State Univ.
Burns, Robert K., Jr.	Univ. of Rochester
Burr, George O.	Univ. of Minn.
Burrows, M. T.	Pasadena, Calif.

Burrows, William	Univ. of Chicago
Burstein, C. L.	N. Y. Univ. Med.
Butcher, E. O.	Hamilton Coll.
Butt, E. M.	Univ. of So. Calif.
Butts, Joseph S.	Oregon State Coll.
Byerly, T. C.	U. S. Animal Exp. Farm, Beltsville, Md.
Byrne, Joseph	Fordham Univ.
C alkins, Gary N.	Columbia Univ.
Cameron, A. T.	Univ. of Manitoba
Cannan, Robert K.	N. Y. Univ.
Cannon, Paul R.	Univ. of Chicago
Cannon, Walter B.	Harvard Med.
Cantarow, Abraham	Jefferson Med. Coll.
Carey, E. J.	Marquette Univ.
Carlson, A. J.	Univ. of Chicago
Carmichael, E. B.	Univ. of Alabama Med.
Carmichael, L.	Tufts Coll.
Carr, J. L.	Univ. of Calif. Hosp.
Carruthers, A.	Birmingham, England
Casey, Albert E.	Louisiana State Univ.
Cash, James R.	Univ. of Va.
Casida, L. E.	Univ. of Wisconsin
Cattell, McKeen	Cornell Univ. Med. Coll.
Cecil, R. L.	Cornell Univ. Med. Coll.
Cerecedo, L. R.	Fordham Univ.
Chace, Arthur F.	N. Y. Post-Graduate Med. Coll.
Chaikoff, I. L.	Univ. of Calif.
Chambers, Robert	New York Univ.
Chambers, William H.	Cornell Univ. Med. Coll.
Chang, Hsi Chun	Peiping Union Med. Coll.
Chang, Hsiao-Chien	Hunan, China
Chargaff, Erwin	Coll. of Phys. and Surg.
Charipper, H. A.	New York Univ.
Cheer, S. N.	W. China Union Univ.
Chen, Graham M.	Univ. of Chicago
Chen, K. K.	Eli Lilly and Co., Indianapolis
Chen, T. T.	Peiping Union Med. Coll.
Cheney, R. H.	Long Island Univ.
Chidester, F. E.	Toronto, Can.
Child, C. M.	Stanford Univ.
Chittenden, R. H.	Yale Univ.
Chouke, K. S.	Univ. of Pa.
Chow, B. F.	Squibb Inst., New Brunswick, N. J.
Christensen, K.	St. Louis Univ.
Christian, Henry A.	Peter Bent Brigham Hosp.
Christman, Adam A.	Univ. of Mich.
Chu, F. T.	Peiping Union Med. Coll.
Chung, H. L.	Peiping Union Med. Coll.
Clark, A. J.	Univ. of Edinburgh, Scotland

Clark, Ada R.	Coll. of Phys. and Surg.
Clark, Guy W.	Lederle Lab., Pearl River, N. Y.
Clark, P. F.	Univ. of Wisconsin
Clarke, Hans T.	Coll. of Phys. and Surg.
Claude, A.	Rockefeller Inst.
Clausen, H. J.	Univ. of Colo. Med.
Claussen, S. W.	Strong Memorial Hosp., Rochester, N. Y.
Clawson, Benjamin J.	Univ. of Minn.
Clifton, Charles E.	Stanford Univ.
Clowes, G. H. A.	Eli Lilly and Co., Indianapolis
Coca, A. F.	Oradell, N. J.
Coggeshall, L. T.	Rockefeller Foundation
Coghill, G. E.	Gainesville, Fla.
Cohen, Barnett.	Johns Hopkins Med.
Cohen, Martin.	N. Y. Post-Graduate Med.
Cohen, Milton B.	St. Alexis Hosp., Cleveland
Cohn, A. E.	Rockefeller Inst., N. Y.
Cohn, Isidore.	New Orleans, La.
Cole, Arthur G.	Univ. of Ill. Med.
Cole, E. C.	Williams Coll.
Cole, Harold H.	Univ. of Calif., Davis
Cole, L. J.	Univ. of Wise.
Cole, Rufus I.	Rockefeller Inst., N. Y. City
Cole, Warren H.	Univ. of Ill. Med.
Cole, William H.	Rutgers Univ.
Collens, William S.	Brooklyn, N. Y.
Collett, Mary E.	Western Reserve Univ.
Collier, William D.	St. Elizabeth's Hosp., Youngstown, O.
Collins, D. A.	Temple Univ.
Collip, J. B.	McGill Univ.
Compere, E. L.	Univ. of Chicago
Conklin, E. G.	Princeton Univ.
Connor, Charles L.	Univ. of Calif. Med.
Cook, Charles A.	Burroughs Wellcome and Co.
Cook, Donald H.	School of Tropical Med., San Juan, P. R.
Cooke, J. V.	Washington Univ.
Coombs, Helen C.	N. Y. Homeopathic Med. Coll.
Cooper, Frank B.	W. Penn. Hosp., Pittsburgh
Cope, O. M.	N. Y. Homeopathic Med.
Copenhaver, W. M.	Columbia Univ.
Corey, E. L.	Univ. of Va.
Cori, Carl F.	Washington Univ.
Corley, Ralph C.	Purdue Univ.
Corner, George W.	Univ. of Rochester
Corper, H. J.	National Jewish Hosp., Denver, Colo.
Co Tui	N. Y. Univ. Med. Coll.
Cowdry, E. V.	Washington Univ.
Cowgill, George R.	Yale Univ.
Cox, Herald R.	U. S. Public Health Inst., Hamilton, Mont.
Cox, Warren M., Jr.	Mead Johnson Co.

Craig, C. F.	Tulane Univ.
Cram, Eloise B.	Nat. Inst. of Health, Washington
Crampton, C. Ward	N. Y. Post-Graduate Med.
Crandall, L. A., Jr.	Univ. of Tenn.
Creaser, C. W.	Wayne Univ. Med.
Crile, George W.	Western Reserve Univ.
Crittenden, Phoebe J.	George Washington Univ.
Crohn, Burrill B.	Mt. Sinai Hosp., N. Y.
Csonka, F. A.	U. S. Dept. of Agr., Washington, D. C.
Cummins, Harold	Tulane Univ.
Cunningham, Bert	Duke Univ.
Cunningham, R. S.	Albany Med. Coll.
Curtis, G. M.	Ohio State Univ.
Curtis, Maynie R.	Columbia Univ.
Cutler, Elliott C.	Peter Bent Brigham Hosp., Boston
Cutting, W. C.	Stanford Univ. Med.
Cutuly, Eugene	Wayne Univ. Med.

D ack, Gail M.	Univ. of Chicago
Dakin, H. D.	Ossining, N. Y.
Dalldorf, Gilbert	Grasslands Hosp., Valhalla, N. Y.
Dalton, A. J.	Western Reserve Univ.
D'Amour, F. E.	Univ. of Denver
Danforth, Charles H.	Stanford Univ.
Danforth, D. N.	Coll. Phys. and Surg., N. Y.
Daniel, J. Frank	Univ. of Calif.
Daniels, Amy L.	Univ. of Iowa
Danzer, Charles S.	N. Y. Homeopathic Med. Coll.
Dautrebande, Lucien	Univ. of Liege, Belgium
Davenport, C. B.	Sta. for Exp. Evolution, Cold Spring Harbor, N. Y.
Davenport, H. A.	Northwestern Univ. Med.
Davis, D. J.	Wilmette, Ill.
Davis, J. E.	Univ. of Vermont
Davis, M. E.	Univ. of Chicago
Dawson, James A.	Coll. City of N. Y.
Dawson, M. H.	Columbia Univ.
Day, A. A.	Northwestern Univ. Med.
Day, Paul L.	Univ. of Arkansas Med.
DeBodo, Richard	New York Univ. Med.
Decherd, George M.	Univ. of Texas Med.
DeEds, Floyd	Stanford Univ. Med.
DeGowin, E. L.	State Univ. of Iowa
DeGraff, A. C.	N. Y. Univ. Med.
Dennis, E. W.	Amer. Univ. of Beirut
DeRenyi, G. S.	Univ. of Pa.
DeSavitsch, Eugene	Washington, D. C.
Detwiler, S. R.	Columbia Univ.
Deuel, Harry J., Jr.	Univ. of S. Calif. Med.
Dick, George F.	Univ. of Chicago
Dickson, E. C.	Stanford Univ. Med.

Dieckmann, W. J.	Univ. of Chicago
Dienes, Louis	Massachusetts General Hosp., Boston
Dieuaide, Francis R.	Brookline, Mass.
Doan, Charles A.	Ohio State Univ.
Dochez, A. R.	Presbyterian Hosp., N. Y. City
Dock, William	Stanford Univ.
Doisy, Edward A.	St. Louis Univ.
Dolley, W. L., Jr.	Univ. of Buffalo
Dominguez, R.	St. Luke's Hosp., Cleveland
Domm, L. V.	Univ. of Chicago
Donaldson, J. C.	Univ. of Pittsburgh
Dooley, M. S.	Syracuse Univ.
Dorfman, Ralph I.	Yale Univ.
Doubilet, Henry	Mt. Sinai Hosp., N. Y.
Dotti, L. B.	New York Med. Coll.
Doull, J. A.	Western Reserve Univ.
Downes, Helen R.	Memorial Hosp., N. Y. City
Drabkin, D. L.	Univ. of Pa.
Dragstedt, Carl A.	Northwestern Univ.
Dragstedt, Lester R.	Univ. of Chicago
Draper, George W.	Columbia Univ.
Draper, William B.	Univ. of Colo.
Dresbach, M.	Philadelphia, Pa.
Drury, D. R.	Univ. of S. Calif. Med.
Dubin, Harry E.	N. Y. City
DuBois, E. F.	Cornell Univ. Med. Coll.
DuBois, F. S.	Hartford, Conn.
Dubos, Rene J.	Rockefeller Inst.
Duggar, B. M.	Univ. of Wisconsin
Dukes, H. H.	Cornell Univ.
Dunn, Leslie C.	Columbia Univ.
Dunn, Max	Univ. of Calif.
Duran-Reynals, F.	Yale Univ.
Dusser de Barenne, J. G.	Yale Univ.
Dutcher, R. Adams	Penn. State Coll.
Duval, C. W.	Tulane Univ.
Du Vigneaud, Vincent	Cornell Univ. Med. Coll.
Dye, Joseph A.	Cornell Univ. Med.
Dyer, Helen M.	George Washington Univ. Med.
Dyer, R. Eugene	National Inst. of Health, Washington
E arle, Wilton R.	National Cancer Inst., Bethesda, Md.
Eastman, N. J.	Johns Hopkins Univ.
Eaton, Alonzo G.	Louisiana State Univ.
Eaton, M. D.	Dept. of Public Health, Berkeley, Calif.
Eberson, Frederick	Alexandria, La.
Ecker, E. E.	Western Reserve Univ.
Eckstein, Henry C.	Univ. of Mich.
Eddy, Walter H.	Columbia Univ.
Edmunds, C. W.	Univ. of Mich.

Edwards, D. J.	Cornell Univ. Med. Coll.
Edwards, John G.	Univ. of Buffalo
Edwards, Philip R.	Kentucky Agri. Exp. Sta.
Eggston, Andrew A.	N. Y. Manhattan Eye, Ear Hosp.
Eichelberger, Lillian	Univ. of Chicago
Ellinger, F. P.	Montefiore Hosp., N. Y.
Ellis, Max M.	Univ. of Mo.
Elsberg, Charles A.	Neurological Inst., N. Y. City
Elser, W. J.	Cornell Univ. Med. Coll.
Elvehjem, C. A.	Univ. of Wisconsin
Emerson, G. A.	West Va. Univ. Med.
Emery, F. E.	Univ. of Buffalo
Emge, L. A.	Stanford Univ.
Enders, J. F.	Harvard Univ.
Engle, E. T.	Columbia Univ.
Epstein, A. A.	Mt. Sinai Hosp., N. Y.
Erlanger, Joseph	Washington Univ.
Ernstene, Arthur C.	Cleveland Clinic
Essex, Hiram E.	Mayo Clinic
Etkin, William	Coll. City of N. Y.
Evans, Alice C.	National Inst. of Health, Washington
Evans, Gerald T.	Univ. of Minn.
Evans, Herbert M.	Univ. of Calif.
Everett, M. R.	Univ. of Okla. Med.
Ewing, James.	Cornell Univ. Med. Coll.
Eyster, J. A. E.	Univ. of Wise.
F aber, Harold K.	Stanford Univ. Med.
Fahr, George	Univ. of Minn.
Falk, K. George	N. Y. Univ. Med. Coll.
Famulener, L. W.	Englewood, N. J.
Farmer, Chester	Northwestern Univ.
Farr, L. E.	Rockefeller Inst.
Faust, Ernest C.	Tulane Univ.
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